

A Longitudinal View of How Noise-Induced Hearing Loss Impacts Auditory and Non-Auditory CNS Activity and the Relationship to Tinnitus Behavior

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Abstract

Tinnitus, defined as the perception of sound when no corresponding external sound is present, affects 50 million people in the United States with 2 million reporting decreased quality of life. Although the etiology of tinnitus is heterogeneous, exposure to a damaging auditory stimulus is the most common cause of the perceptual disorder. In addition to the better known auditory component of tinnitus there is an affective component. Anxiety and depression can occur concomitantly with tinnitus and is often of unknown etiology. Exposure to damaging sound leads to complex changes throughout the central nervous system (CNS) impacting both auditory and non-auditory brain areas. The absence of a *complete* picture of how tinnitus is manifested and maintained in the CNS continues to hinder the development of effective treatments. The goal of this project is to elucidate the underlying mechanisms that produce neuroplastic changes over time in the central nervous system following sound damage that may or may not be associated with tinnitus. Using an animal model of sound induced tinnitus, this project evaluates both early and long-term changes in behavior, neuronal activity, and early changes to neuroplastic protein marker expression in various auditory and non-auditory brain regions. The findings reported here reveal information about the timeline of peripheral injury (sound damage) to tinnitus onset and changes that take place in six different brain regions encompassing both auditory and non-auditory brain regions. This project has allowed us to enhance our understanding of the development of tinnitus over time in several auditory and non-auditory brain structures at both the molecular and systems level in addition to obtaining corresponding changes in behavior.

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Chapter 1: Introduction

1.1 Tinnitus Epidemiology and Etiology

Tinnitus, the perception of sound in the absence of external auditory stimuli, affects 50 million people in the United States according to the American Tinnitus Association (ATA.org), and a subpopulation of tinnitus sufferers are debilitated. Tinnitus has been described as occurring in two forms; objective and subjective. The less common form of tinnitus is objective and consists of a generated sound that can be heard and observed by both patient and physician. Subjective tinnitus is more common and experienced by the patient alone (Minen et al 2014). The etiology of tinnitus is heterogeneous, with the most common cause being recreational, occupational and firearm noise exposure (Agrawal et al 2009). Tinnitus impacts a wide range of the population from young adults to older individuals (Loprinzi et al 2013, Vogel et al 2014). It has been shown that women experience more complex tinnitus than men (Dineen et al 1997) but overall tinnitus is more common among men (Seidman et al 2010). Warfighters returning from Iraq and Afghanistan often suffer from tinnitus, and the cost for seeking clinical help has been staggering (Helfer et al 2011). The prevalence of tinnitus among military personnel is high, with 3-4 million veterans presenting with tinnitus and 1 million seeking clinical treatment (ATA). Hundreds of millions of dollars are paid out per year in disability compensation alone to individuals suffering from tinnitus (Saunders & Griest 2009).

Many patients with tinnitus also present with neuropsychiatric symptoms such as depression and anxiety, often of unknown etiology (Cho et al 2013, Joos et al 2012, Minen et al 2014). Clinically, it has been reported that approximately 70% of patients with tinnitus suffer from emotional distress (Gomaa et al 2013, Holmes & Padgham 2011, Shargorodsky et al 2010). However, the relationship between tinnitus and neuropsychiatric disorders is not well

understood. A recent study evaluated depression levels in patients with moderate to severe tinnitus and found that depressive symptoms were observed in 57% of those evaluated (Cho et al 2013). Gomaa and colleagues (2013) have suggested that tinnitus alone, and not hearing loss, is the dominant factor in causation of depression. Depression itself is a devastating illness that can be costly and debilitating, with an estimated lifetime prevalence of 17% in the United States (Donohue & Pincus 2007, Kessler et al 1994, Wang et al 2003). Both chronic tinnitus and depression can result in disruption to activities of daily living, poor social outcomes and loss of work productivity. These extraordinary monetary costs highlight the economic burden to society and the federal government and emphasize the importance of studying the mechanisms of tinnitus as a means to develop effective treatments that considers all aspects of the disorder.

1.2 Treating Tinnitus

While tinnitus is becoming more common, the pathology is not well understood. Tinnitus research has provided some insight into the mechanism of tinnitus development; however there are still gaps that need to be filled in order to develop effective treatments. Currently, there is no existing therapeutic intervention that is effective for all tinnitus patients.

External noise has long been utilized to help individuals manage their tinnitus in many different capacities (e.g. sound creating devices, hearing aids). The effectiveness of sound therapy alone has proven to be inconclusive (Hobson et al 2012). Sound therapy in conjunction with intensive educational counseling, also called Tinnitus Retraining Therapy (TRT), has helped many patients. TRT was developed based on the neurophysiological model of tinnitus which highlights that all levels of the auditory and non-auditory systems may have a

role in tinnitus, emphasizing a non-auditory role in degree of tinnitus annoyance (Jastreboff 1990). The goal is to induce and facilitate habituation to the tinnitus signal in the patient. Although an individual's perception of tinnitus may be unchanged when in focus, their annoyance is decreased and the patient is otherwise unaware of their tinnitus (Jastreboff et al 1996). The results of a survey of more than 100 individuals with tinnitus by Jastreboff and colleagues (1996) showed greater than 80% improvement when patients received counseling in combination with use of noise generators.

Cognitive Behavioral Therapy (CBT), a form of psychotherapy with focuses on modifying dysfunctional thought patterns and behavior, has had variable effectiveness. CBT as a treatment for tinnitus addresses an individual's reaction to tinnitus. One meta-analysis found that CBT was shown to improve quality of life and depression scores but not tinnitus loudness perception in individuals with subjective tinnitus (Martinez-Devesa et al 2010). A randomized controlled study reported that CBT in a group format utilizing a manual is effective at alleviating negative emotional distress associated with tinnitus (Robinson et al 2008). In some cases psychotherapeutic interventions were shown to only be effective when therapy was ongoing or for a short duration after therapy (Frank et al 2006)

Pharmacological agents have been used to treat tinnitus, proving to be effective for some individuals. SSRIs in conjunction with psychotherapy were shown to bring significant relief of tinnitus symptoms to a population of individuals suffering from both tinnitus and depression (Folmer & Shi 2004). Thirty-two percent of individuals with tinnitus reported improved symptoms while taking clonazepam (Gananca et al 2002). Treatment with the antidepressant, cyclobenzaprine achieved partial to complete suppression of tinnitus behavior in a sound induced tinnitus rat model (Lobarinas et al 2015).

In recent years, the use of therapy intended to induce targeted neural plasticity to treat tinnitus has gained popularity as it has the potential to restore brain activity in circuits that are disrupted in tinnitus (Engineer et al 2013). In a group of individuals with treatment resistant tinnitus, repetitive transcranial magnetic stimulation (rTMS) directed to the auditory cortex was shown to significantly reduce tinnitus severity (Anders et al 2010). Engineer and colleagues (2011) reported elimination of physiological and behavioral evidence of tinnitus that lasted for weeks after brief pulses to the vagal nerve paired with tones (Engineer et al 2011). While transcranial magnetic stimulation has proven to be an effective treatment, questions remain about its usability in everyday practice (Londero et al 2018).

There are two components of chronic tinnitus (perceptual and affective) and treatments that address both the perceptual (elimination or reduction of tinnitus percept) and affective components (treat response to tinnitus and address quality of life) are likely to be more effective.

1.3 Tinnitus Research Past and Present

One of the first documented cases of tinnitus was mentioned in the Greek Hippocratic Corpus where the tinnitus percept is described as “ringing”. Explanation for this phenomenon, as written in this ancient document, was described by humoral theory whereby an excess amount of heat and blood in the head was thought to induce swelling of the auditory cavity (that would otherwise be filled with air), influencing a patient’s perception of sound (Maltby 2012). Fast forward through time several centuries to 1961 when Georg Von Békésy was awarded the Nobel Prize in Physiology or Medicine for his work in the auditory system. Békésy helped pave the way for auditory neuroscientists when he discovered that the cochlea housed sensory

receptors capable of transmitting sound waves through neural fibers from the peripheral to central nervous system. The understanding of cochlear mechanics and sound transduction enabled researchers to study different aspects of auditory sensation and perception.

Originally proposed hypotheses posited that tinnitus was a symptom resulting from damage to the auditory periphery that was maintained solely in the periphery. Under this assumption it is logical to think that eliminating passage of signal from the auditory periphery to the central nervous system would relieve tinnitus percepts. However, surgical treatment has not been shown to eliminate the tinnitus percept (Berliner et al 1992, House & Brackmann 1981). More specifically, elimination of auditory input from the periphery, following transection of the vestibulocochlear nerve, relieved tinnitus in 45% of patients tested while 55% reported no change or worsened tinnitus post operatively (House & Brackmann 1981).

A paradigm shift has been made since tinnitus researchers determined that tinnitus perception is not solely a result of aberrant sensory information from the periphery. Rather, it is a phenomenon that begins in the auditory periphery and precedes a cascade of changes throughout central auditory and non-auditory brain regions. Animal studies have shown that noise and drug induced hearing loss and tinnitus is often accompanied by changes in spontaneous neural activity and protein expression in various auditory brain regions (Baizer et al 2012, Brozoski et al 2013, Dong et al 2010, Kennon-McGill 2014, Mazurek et al 2012). Common sites of these central changes are the dorsal cochlear nucleus (Kaltenbach & Afman 2000, Kaltenbach et al 2002, Kaltenbach et al 2005), inferior colliculus (Bauer et al 2008, Dong et al 2010), and primary auditory cortex (Norena & Eggermont 2005, Seki & Eggermont 2003). Central auditory structures can be affected following sound damage, with tinnitus being the

result approximately 50% of the time (Engineer et al 2011, Kraus et al 2010, Pace & Zhang 2013).

In more recent years it has become evident that the disorder is more complex, involving additional brain areas including, but not limited to, the cerebellum, hippocampus and amygdala (Bauer et al 2013a, Bauer et al 2013b, Brozoski et al 2007, De Ridder et al 2011, Kraus & Canlon 2012). Neuroimaging studies in the clinical tinnitus population have found consistent pathophysiological changes in limbic brain regions, including the amygdala, hippocampus, and anterior cingulate cortex (De Ridder et al 2013, Landgrebe et al 2009). More importantly, structural and functional changes in the auditory and limbic system are strongly correlated in tinnitus patients (Leaver et al 2011). While changes to non-auditory brain regions have been observed in tinnitus patients and animal models of tinnitus, their contribution to the disorder is not well understood.

In fact, the majority of recent research has implicated many auditory and non-auditory brain regions in the onset and maintenance of the disorder making this a multifaceted problem (Kraus & Canlon 2012, Leaver et al 2011, Rauschecker et al 2010). Changes that ultimately result in tinnitus appear to occur along a timeline affecting several anatomical structures; the peripheral auditory system, central auditory system, the limbic system and higher-order brain structures (Brozoski & Bauer 2005, Brozoski et al 2013, Brozoski et al 2012, Duan et al 2000, Eggermont 2006, Eggermont & Roberts 2004, Guitton 2012, Guitton & Dudai 2007, Kaltenbach & Afman 2000, Norena & Eggermont 2003). Cochlear application of NMDA antagonists prior to sound damage prevents tinnitus development (Duan et al 2000). Additionally, blockade of NMDA receptors within a brief time window post sound damage, can prevent chronic tinnitus (Guitton & Dudai 2007). Altered spontaneous firing rate (SFR) is

evident in the auditory cortex just a few hours after acoustic trauma (Norena & Eggermont 2003) while changes in SFR are not detected in the DCN until several days later (Kaltenbach & Afman 2000). Onset of tinnitus is prevented by bilateral ablation of the DCN prior to sound damage (Brozoski et al 2012). However when the ablation was done post sound damage, animals displayed behavioral evidence of tinnitus (Brozoski & Bauer 2005).

These studies suggest that acute and chronic development of tinnitus are mechanistically distinct and have unique brain changes associated with them. Investigating underlying mechanisms of tinnitus onset in auditory and limbic brain regions and the changes in neuronal activity that manifest over time will provide a foundation by which to develop effective treatments for chronic tinnitus.

1.4 Tinnitus Induction

High level sound exposure, salicylate, other ototoxic drugs and aging result in differing degrees of damage in the peripheral auditory system and a common symptom among all is tinnitus. Salicylate and noise damage are the most common methods used to induce tinnitus in animal research. High doses of salicylate, the active compound in aspirin, can cause temporary hearing loss and high pitch tinnitus in both humans and animals (Groschel et al 2016). Stolzberg et al. (2012) reported decreased sensitivity of sensory hair cells and modulation of spontaneous firing of the auditory nerve as a result of salicylate toxicity. The major benefit of this method to auditory researchers is that it leads to rapid onset of tinnitus behavior that is reversible (Stolzberg et al 2012). Additionally, salicylate induced tinnitus is reliable (Ralli et al 2010) unlike noise exposure that produces variable tinnitus (approximately 50% of animals) within a population of animals that were exposed to the same sound

damaging stimulus (Kraus et al 2010, Pace & Zhang 2013). The use of high doses of salicylate in animal models has allowed auditory researchers to investigate the biological mechanisms that occur with tinnitus within a short duration of time.

While salicylate induced tinnitus has its benefits, one of the most common causes of subjective tinnitus in the human population is exposure to loud (intense) sounds. The salicylate induction method provides information about the underlying mechanisms of rapid and reversible tinnitus (acute tinnitus) but lacks the longevity required to study later onset, lasting tinnitus (chronic tinnitus). The variability inherent in the sound induced tinnitus model more closely mimics the human experience. Many individuals in the clinical tinnitus population also suffer from some degree of hearing loss resulting from exposure to damaging sounds. Additionally, sound damage and hearing loss does not reliably lead to tinnitus in humans. Tinnitus is not a guaranteed symptom resulting from peripheral damage induced by sound in either the human or animal research populations. Cross study comparisons are made more difficult due to differences in the details of the sound damaging stimulus (e.g. intensity, frequency and duration) and their impact on hearing loss.

1.5 Identifying Tinnitus Behavior

Animal models of tinnitus are important as they allow investigators to control for the additional variability inherent in human studies (e.g. age, ethnicity, gender, presence of other disorders). Because peripheral damage or hearing loss does not always result in the symptom of tinnitus, it is important to distinguish between findings evident of hearing loss with and without tinnitus. Therefore, auditory researchers have developed three paradigms that can be used to identify tinnitus behavior in animals. It's important to note that animal models of

tinnitus induce some degree of hearing loss therefore any behavioral assessment should consider the possible confound of the effects of hearing loss. Paradigms used to measure tinnitus behavior can be grouped into three categories: Conditioned avoidance, positive reinforcement and gap-startle reflex (von der Behrens 2014).

Jastreboff and colleagues (1988) utilized a conditioned suppression task to identify tinnitus behavior in a salicylate animal model of tinnitus. During training animals were exposed to a background noise during which time they were allowed to drink water. The conditioned stimulus (CS) was a period of silence in the otherwise constant background noise. A suppression ratio (ratio of number of licks during CS/number of licks during background sound preceding the silent period) was collected for each animal. During suppression training the CS always ended with a foot shock (unconditioned stimulus US) that eventually led to extinction of licks during the silent period. Successfully trained animals were identified as “tinnitus behaving” post salicylate treatment if they continued to lick even during the CS, the idea being that tinnitus does not allow for detection of silence (Jastreboff et al 1988, von der Behrens 2014). Bauer and Brozoski (2001) developed another version of conditioned avoidance that relies on the psychophysical features of auditory stimuli and an animal’s detection ability. They have shown its use in both salicylate and noise induced tinnitus models. Animal subjects were placed on a restricted diet and animals were trained to press a lever to receive food. They were then conditioned to cease lever pressing during periods of silence and a suppression ratio was calculated. During training, suppression ratios of ≥ 0.2 resulted in a foot shock at the end of the silent period. This training conditioned animals to respond accordingly during noise (lever press resulted in food but not aversive stimuli), tones (lever press resulted in food but not aversive stimuli) or silence (lever presses resulted food pellet in addition to a foot shock).

Testing phases occurred once animals were responding reliably, which usually occurred after 12-15 trials. Again, animals unable to detect silence are identified as tinnitus behaving (Bauer & Brozoski 2001). An added benefit to this method that differs from the Jastreboff method is that it closely parallels tinnitus testing in humans as it allows researchers to gather tinnitus pitch and loudness information in addition to simple tinnitus identification. However this testing paradigm requires careful behavioral training, and it can take several months before animals are ready for the testing phase (von der Behrens 2014).

Another paradigm used by Ruttiger et al. (2003) to identify tinnitus behavior post salicylate administration minimized the need for aversive stimuli (foot shock) and rather emphasized positive reinforcement to train animals. For this test, rats were trained to access two drinking spouts filled with 3% sucrose (reward) in the presence of an auditory stimulus. Animals were required to access both spouts to collect a reward. During periods of silence, animals that drank from only one spout were not rewarded but rather received a weak foot shock. When testing for tinnitus there were no rewards or punishments during silent periods. The behavioral readout was the ratio between activity (accessing reward spout) during noise and during silence divided by the ratio between duration of noise and duration of silence. Tinnitus was evident in animals that accessed reward spouts in silence relative to noise presentation (Ruttiger et al 2003, von der Behrens 2014).

Behavioral tests that could identify tinnitus without extensive training were desirable for experiments examining tinnitus on a short timeline. The most widely used gap-startle reflex paradigm developed by Turner et al. (2006) is based on the naturally occurring startle reflex and therefore does not require a training period. Gap-startle reflex testing is based on the acoustic startle reflex (ASR) that causes muscles to contract rapidly when the animal is

startled by a loud sound. During a testing session the animal is placed in a small chamber equipped with a Piezo transducer in the floor. Figure 1 shows a rat in a startle chamber in addition to a schematic of presented trial types. Animals are exposed to a continuous background sound centered at a specific frequency that includes two trial types: gap (silent gap precedes startle stimulus) and no gap (startle only, no silent gap). Behavioral readout is the amplitude of force applied on the floor during the startle pulse and the animal's ability to inhibit the startle reflex during gap trials. If an animal has tinnitus then it is assumed that it cannot detect the gap (due to tinnitus perception) and therefore there is no inhibition of the startle reflex (Turner et al 2006, Turner & Parrish 2008, von der Behrens 2014). This behavioral paradigm has many benefits in that it does not require extensive training or food/water restriction, resulting in relatively high-throughput screening for tinnitus behavior. However, as with most behavioral testing paradigms used in animal research, there are limitations. Lobarinas et al. (2013) suggested that hearing loss may influence gap detection results and designed a study to control for this potential confound. In this study, the startle pulse was optimized so that the intensity of the stimulus was outside the range of hearing loss that occurred with tinnitus induction. The presentation of a rapid air puff to the animal's back replaced the broadband noise startle stimulus. It was reported that even in cases of conductive hearing loss, the startle reflex was preserved with the air puff approach (Lobarinas et al 2013a). During the initial development of the gap-startle paradigm, a variety of control procedures were done to demonstrate the results are due to tinnitus perception and not hearing loss or some other factor (Turner et al 2006). Another way around the problem of hearing loss is to induce tinnitus unilaterally, leaving one ear with intact hearing. Use of auditory brainstem response measurements following damage ensures that each cohort of

damaged animals can detect the startle stimulus. Another potential downfall of gap detection testing is translatability, as it is not known if the tinnitus percept would fill in periods of silence in the human population. Ideally, a more objective measure of tinnitus in animals could also be utilized to measure tinnitus objectively in human subjects with tinnitus. Fournier and Hebert (2013) designed a study to measure startle response (eye blink) in humans with high pitched tinnitus and compared their findings to animal studies. They concluded that tinnitus patients had similar response amplitudes to damaged (tinnitus) animals when the startle was preceded by a silent gap (Fournier & Hebert 2013). The findings of this study suggest that gap detection may be a useful tool to objectively detect tinnitus in both human and animal populations.

While measuring tinnitus in animal models has its challenges, if we want to determine brain changes associated with tinnitus then the use of one paradigm or another is necessary. Optimization parameters both within the testing sessions and data analysis of the gap startle paradigm as a means of increasing its reliability and usability are presently being explored and reported by several investigators (Lobarinas et al 2013b, Longenecker & Galazyuk 2012).

Research has shown that both auditory and non-auditory brain areas can be affected in individuals with tinnitus, including limbic brain regions (Chen et al 2015, De Ridder et al 2006, Gunbey et al 2017, Joos et al 2012, Rauschecker et al 2010). The limbic system is the emotional control center of the brain (Balleine & Killcross 2006, LeDoux). Individuals with tinnitus often have comorbid depression and anxiety. Animal models of anxiety have shown that the open field test can be used to measure anxiety in rodents (Ramos et al 2008, Seibenhener & Wooten 2015). The behavioral readout of this test allows investigators to determine how much time an animal spends in the center of an open field (testing arena) vs. time spent in the surround (perimeter around the arena). This test is based on thigmotaxis, the

tendency to remain close to walls when allowed to explore a given area. Measurements of thigmotaxis can be used to assess anxiety in rodents (Simon et al 1994). The Open Field Test (OFT) involves placing an animal in an open field arena with designated regions that correspond to “center” vs. “surround” allowing quantification of time spend in each region. Animals that spend more time exploring the perimeter or “surround” of the arena relative to baseline are considered to be displaying anxiety behavior. In contrast, animals that explore the entire arena and spend a majority of testing time in the center are thought to be void of anxiety.

1.6 Elucidating Tinnitus Generators

In order to develop effective treatments it is imperative that we understand the site(s) where tinnitus is being generated and also maintained in chronic tinnitus. This issue has proven to be more complex in recent years as changes in every brain region along the central auditory pathway can be impacted by the perceptual damage that induces tinnitus. The connection within the central auditory system and how sound is perceived must be understood before tinnitus generators can be identified (Figure 3).

Mechanical energy in the form of sound waves enters the auditory canal and vibrations of the tympanic membrane are transferred along the basilar membrane. This mechanical energy ultimately results in depolarization of sensory hair cells inside the cochlea. Basilar membrane movement deflects stereocilia on the apical surface of the hair cell, and ions flow across the cell membrane through mechanically gated ion channels. The change in membrane potential of the hair cell leads to the release of neurotransmitters at their basal surface richly innervated by spiral ganglion cell terminals. The resulting action potential

transmits the signal via the vestibulocochlear nerve (eighth nerve). Hair cells are tonotopically arranged with high frequency responders at the basal end of the cochlea and low frequency responders located at the apex of the cochlea. This tonotopy is preserved in some fashion throughout the entirety of the central auditory system.

Figure 3 illustrates both peripheral and central auditory structures. Each structure can be considered part of a direct pathway for perception of auditory information, or as having a more indirect effect on auditory processing. In the direct pathway, sound information originating in the given cochlea travels through the auditory nerve and synapses in the ipsilateral cochlear nucleus. The cochlear nucleus (CN) can be divided into two sub regions; dorsal cochlear nucleus (DCN), ventral nucleus (VN). Both the DCN and VN have been implicated in tinnitus generation due to direct interaction with the periphery via the auditory nerve (Chang et al 2002, Neal 2016, Shore et al 2008, Tzounopoulos 2008). There are commissural connections that allow for communication between the ipsilateral and contralateral CN (Brown et al 2013). Information from the CN projects bilaterally to the superior olivary complex (SOC) which supports sound localization and contralaterally to the inferior colliculus (IC) for perception of sound.

The inferior colliculus is the major auditory processing center in the midbrain and can be divided into three sub regions: central nucleus, dorsal cortex and external nucleus. The inferior colliculus has been widely studied in tinnitus research due to its ascending and descending interactions with other auditory nuclei (Bauer et al 2008, Imig & Durham 2005, Kennon-McGill 2014, Knipper et al 2010). The contralateral inferior colliculus is the next obligatory synapse from the CN en route to the auditory cortex in the direct pathway.

Within the direct pathway, ascending projections from the IC extend ipsilaterally to the auditory thalamus (medial geniculate body [MGB]). In recent years the MGB has become an additional site of interest in tinnitus studies due to its unique position to gate sound perception as it projects to the auditory cortex (Caspary & Llano 2017, Gunbey et al 2017, Kalappa et al 2014).

Neural signals from the MGB project on to the final obligatory synapse in the central auditory pathway, the ipsilateral auditory cortex, where sound perception occurs. Located in the temporal lobes, it is divided into three sub regions; the primary cortex, secondary cortex and association areas. While the auditory cortex is known to be impacted in tinnitus, the complex organization of the cortex makes it a difficult region to study. The borders within the auditory cortex are ill defined and each region contains differing tonotopy. Peripheral auditory damage has been shown to induce large amounts of tonotopic organization which adds to the complexity of studying its association with tinnitus (Huetz et al 2014, Seki & Eggermont 2003, Zhang et al 2011).

Further complicating tinnitus research is the involvement of additional non-auditory brain regions. Areas within the limbic system, including the amygdala and hippocampus have been shown to be impacted in both human and animal studies of tinnitus (Gunbey et al 2017, Kraus & Canlon 2012, Kraus et al 2010, Lockwood et al 1998, Seydell-Greenwald et al 2014). Additionally, the flocculus and parafloccular lobe, areas known to be involved in gaze-related motor control (vestibulo-ocular reflex, VOR) within the cerebellum have been shown to be affected in tinnitus models (Bauer et al 2013a, Brozoski et al 2017, Manohar et al 2012, Mennink et al 2018). Lastly, Shore and colleagues have shown that tinnitus also involves the somatosensory system (Dehmel et al 2008, Shore et al 2008, Wu et al 2015). Cochlear

damage results in increased synaptic projections from somatosensory nuclei to auditory structures. Thus, the task of identifying specific sites of tinnitus generation and maintenance is a difficult one and it's likely that tinnitus perception results from changes within a complex network of brain interactions rather than changes within discrete regions. In these experiments we have focused on three auditory (dorsal cochlear nucleus, inferior colliculus, auditory cortex) and three non-auditory (cerebellar parafloccular lobe, hippocampal dentate gyrus, basolateral amygdala) brain regions.

1.7 Evaluation of CNS Plasticity

The complexity of tinnitus research is rife with challenges and while many studies inspire more questions than answers, researchers continue study of this elusive disorder. Altered neuroplastic mechanisms that result from peripheral damage and often result in tinnitus are studied using a number of methods. A key aspect of functional changes thought to be related to tinnitus is spontaneous neural activity. Spontaneous activity in a sensory system is defined as neural activity that occurs in the absence of an external stimulus and is often referred to as resting-state activity (Kutsarova et al 2017). Disrupted spontaneous brain activity as a result of peripheral damage has been shown in multiple brain regions following tinnitus induction and continues to be studied today.

Electrophysiology is the most commonly used method to study spontaneous brain activity in tinnitus models. Single unit (data from a single discharging neuron) and multiunit (data from a group of discharging neurons) electrophysiological recordings have been utilized. Electrophysiological tinnitus studies have mostly reported increased spontaneous firing rate in auditory brain areas following damage: DCN (Brozoski et al 2002, Kaltenbach & Afman 2000),

IC (Kennon-McGill 2014) and auditory cortex (Norena & Eggermont 2003, Seki & Eggermont 2003).

Another method that has been employed as a measure of spontaneous activity is the ¹⁴C-2-deoxyglucose (2DG) assay (Imig & Durham 2005). 2DG is a radioactively labeled modified glucose molecule. The modification allows for 2DG to be taken up into active brain cells but rather than undergo further glycolysis, the molecule accumulates and can then be measured from an autoradiograph that includes several regions of interest. The use of this technique in tinnitus research is becoming less common but its use in conjunction with other methods proves useful in elucidating changes in multiple types of brain activity (Kennon-McGill 2014).

The aforementioned methods of measuring spontaneous activity have inherent challenges. Electrophysiology provides high resolution detail about the activity of only a discrete subset of cells, which is true of both single and multiunit recording procedures. Additionally, surgical procedures to expose regions for exploration are often required, thereby requiring specific surgical training. Electrophysiological results have been shown to differ depending on whether the animal was awake and freely moving or under anesthesia during recordings (Kennon-McGill 2014, Ma & Young 2006). While recordings have been done in awake, freely moving animals, few investigators have done these experiments and the usability for long term, repeated measures is limited (Kennon-McGill 2014). 2DG requires the animal to be euthanized within a short time frame post injection; therefore this method does not allow for measures at multiple time points. Because tinnitus is a complex problem that impacts many brain areas and onset occurs at variable time points post damage, it would be

useful to measure changes in spontaneous activity in multiple brain areas at multiple time points.

A recent tool utilized in tinnitus research, manganese-enhanced MRI (MEMRI) can be used to measure global brain changes longitudinally. This method requires manganese injection followed by an MRI brain scan. Manganese serves as a calcium analog that enters active brain cells via voltage gated calcium channels. Both manganese injection and exposure to the desired auditory environment occur prior to the scan, bypassing any chance of scanner noise influencing contrast enhancement in the output images. Additionally, manganese diffuses out of brain cells over time thereby making it a useful tool to measure changes at multiple, discrete time points (Cacace et al 2014). While many investigators studying tinnitus have utilized this method at either early or late time points post damage, there has not been a long term study done in the same animal cohort which would elucidate information about brain activity changes over time in animal models of tinnitus. Images of animal set up pre-MRI can be viewed in Figure 4.

Immunohistochemistry (IHC), has been used to visualize a variety of protein markers, thought to reflect neuroplasticity. The polysialated form of the neuronal cell adhesion molecules (PSA-NCAM) has been suggested to decrease adhesive forces of the molecule, potentially allowing for migration and reorganization of neurons (Gascon et al 2007, Kiss & Rougon 1997). Expression of PSA-NCAM has also been associated with neurogenesis in the developing brain and neurogenesis, migration, and synaptic plasticity in the adult brain (Bonfanti 2006, Markram et al 2007). Another potential marker of plasticity is growth-associated protein 43 (GAP-43), which is expressed in early brain development but is downregulated as the brain matures. GAP-43 has been shown to reemerge during CNS

reorganization such as neurite outgrowth, restructuring of neurons, and synaptic formation (Rosskoth-Kuhl & Illing 2014). Unilateral cochlear ablation in rats results in increased GAP-43 expression of the cochlear nucleus complex ipsilateral to the damaged ear, with a transient increase also observed in the contralateral side (Illing et al 1997). Noise exposure has been shown to induce reemergence of GAP-43 expression in the ipsilateral inferior colliculus and ventral cochlear nucleus (Michler & Illing 2002). IHC has been used measure other proteins associated with synaptic remodeling (Shore et al 2016, Wu et al 2015).

Doublecortin (DCX), a microtubule associated protein exclusively expressed in neuronal tissue, has been used as a marker of migrating and immature neurons (Friocourt et al 2003, Gleeson et al 1999) and has been associated with neurogenesis (Ernst et al 2014, Klempin et al 2011). DCX has been shown to be elevated in unipolar brush cells (UBCs) (Bauer et al 2013b), DCX expressing excitatory glutamatergic neurons found in the fusiform layer of the DCN and the flocculus and paraflocculus of the cerebellum (Manohar et al 2012), two brain regions implicated in tinnitus. Paolone et al. (2014) investigated whether DCX in these non-neurogenic brain regions signifies neurogenesis by co-labeling cells with DCX and BrdU. While they identified BrdU labeled cells in the brainstem and cerebellum; the numbers and distribution of labeled nuclei did not support the hypothesis that DCX was labeling newly generated cells (Paolone et al 2014). Additionally, a study by Kraus et al. (2010) observed decreased DCX labeling in the hippocampal dentate gyrus (a limbic region implicated in tinnitus) of animals that had been exposed to damaging sound. Taken together, these results suggest that DCX is labeling neuroplastic processes which may include hyperactivity in various central auditory brain regions, a proposed neural correlate of tinnitus. However, DCX labeling

in the hippocampus may be indicative of decreased neurogenesis as a result of peripheral (sound) damage.

1.8 Summary

In the experiments presented here we exposed young adult rats to mild sound to induce hearing loss and tinnitus. To better understand specific neuroplastic changes underpinning hearing loss and tinnitus, we utilized immunohistochemistry to detect doublecortin (DCX) protein in the dorsal cochlear nucleus, cerebellar paraflocculus and the dentate gyrus of the hippocampus of sound damaged animals. We utilized non-invasive manganese-enhanced MRI at acute and chronic time points to evaluate changes in spontaneous activity in three auditory and three non-auditory brain regions. Progression of neurological changes was assessed over time within the same animal. We also measured hearing loss, tinnitus behavior and anxiety behavior in each animal at both time points, allowing us to evaluate relationships between hearing loss, tinnitus and brain activity in auditory and non-auditory brain regions. Data obtained from these measures has provided novel insight into the integrative effects of sound damage in multiple systems; auditory and limbic.

Figure 1. Gap Detection Startle Box and Trial Types with Representative Behavioral Readout

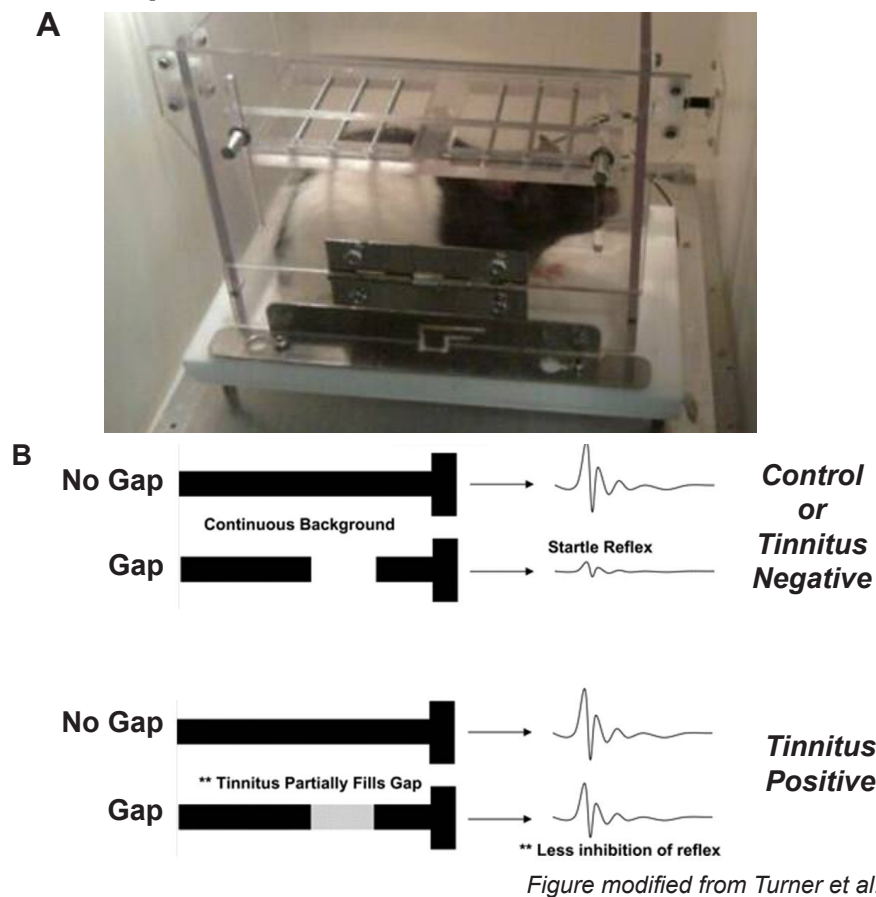


Figure 1. Gap detection testing chamber and schematic of stimuli with subsequent behavioral readout. Positioning of animals within the startle box is shown in A. The acrylic box is located inside of a sound attenuated box. The top of the startle box can be adjusted accordingly for each animal to minimize movement not specific to the test. Two speakers (not shown) used to present background and startle stimuli are positioned above the animal's head. The animal is standing on a forceplate (white surface) equipped with a transducer to measure force applied by the animal. Trial types presented to animal's during testing are shown in B. Startle response amplitudes are shown to the right. The top shows a typical response in a control or tinnitus negative animal. The bottom shows a typical response in a tinnitus positive animal. Expected response amplitude based on presence or absence of tinnitus are shown as amplitude of force applied when startled. Animals were tested over 4 baseline sessions and 4 post-exposure sessions. Background tone stimuli were 1 kHz bands centered at 12, 16, 20 kHz. The duration of the gap was 100 ms, occurring 100 ms before the startle pulse (118 dB SPL for 50 ms). For each background tone, 12 No-Gap trials (startle only) are pseudorandomly intermixed with 10 Gap trials (22 trials per background, 66 trials total). Baseline and post-exposure "gap/no-gap" ratios are calculated for each rat individually. Subsequently, a final gap score is calculated as follows [GD score = Post Exposure (Gap/No-Gap) / Baseline (Gap/No-Gap)].

Figure 2. Open Field Test Behavioral Readout

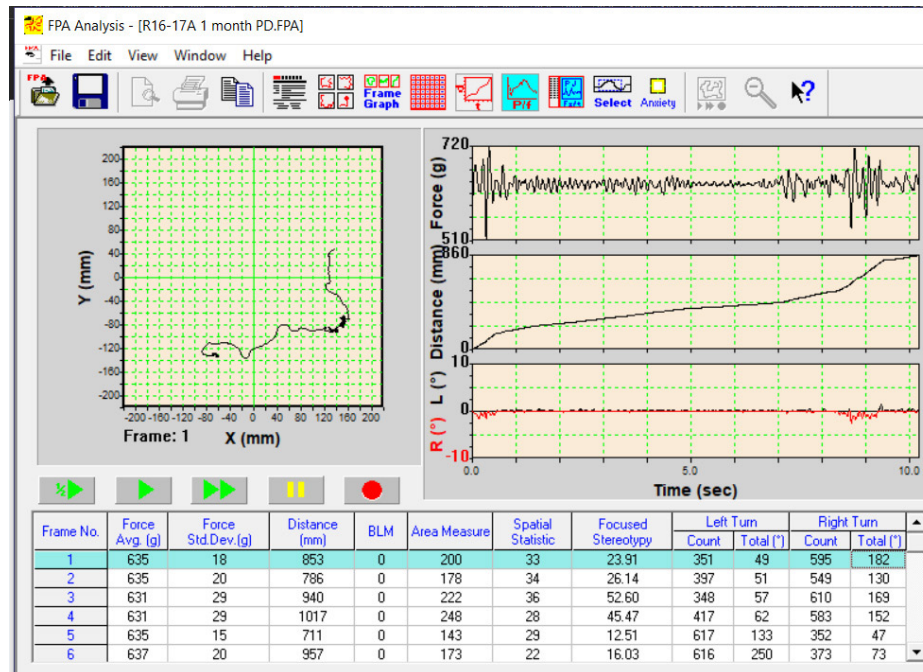


Figure 2.

An example of the behavioral readout used for open field testing (OFT) analysis. The Force Plate Actimeter (BASi, West Lafayette, IN) was used to measure animal location during exploratory behavior within an open field, with less time spent in central areas an indicator of anxiety. The actimeter arena consists of a 356 mm square, stiff horizontal plate attached at the corners to force transducers. A Plexiglas enclosure rests a few millimeters above the plate to create a transparent enclosure. Animals were placed into the testing arena and allowed to move freely for 5 minutes. During this time, the actimeter recorded several parameters such as total distance traveled, area traveled, time spent in the center (200 mm x 200 mm), time spent in perimeter (100 mm strip around the perimeter of the arena). Time spent in the center and perimeter was exported from testing software to Excel for further analysis. Percentage of time spent in the perimeter was calculated ($\% \text{ perimeter} = \text{time spent in perimeter (s)} / \text{total time (s)} * 100$) and plotted as function of time point. Difference scores from baseline for 1 month and 3.5 months post damage to baseline were calculated and z-scores were generated and used for correlation analysis.

Figure 3. Depiction of Peripheral and Central Auditory Structures and Illustration of Direct and Indirect Pathways of the Auditory System

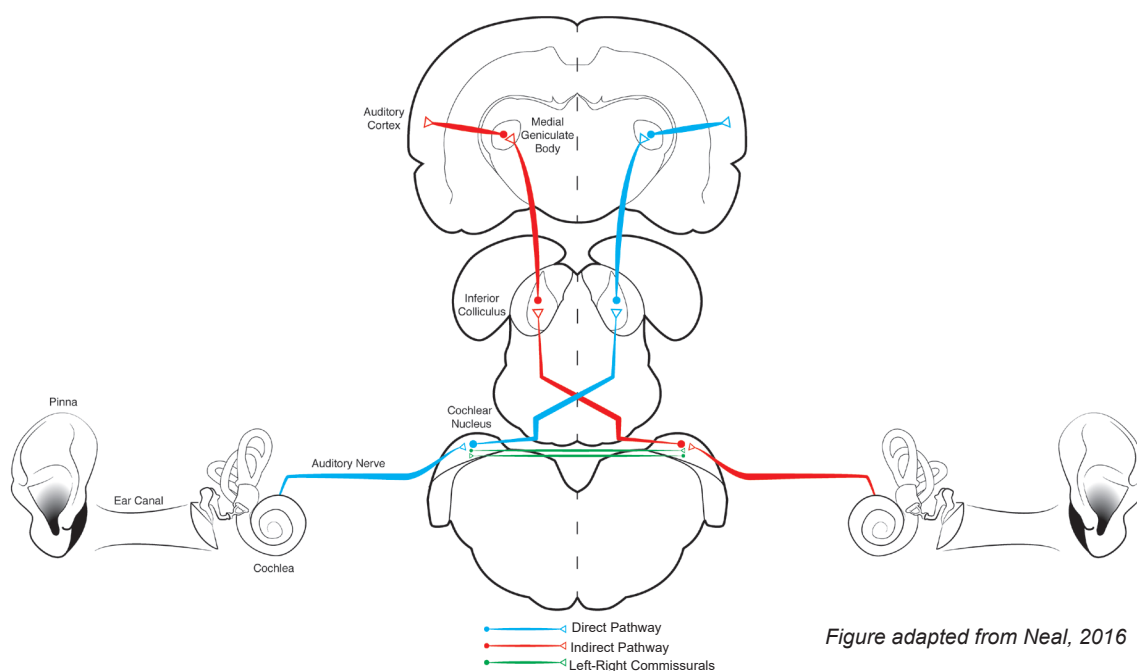


Figure 3. *Simplified diagram of the auditory system. Major structures of the auditory system are depicted in this simplified diagram. The direct pathway connections are represented from the damaged cochlea (shown in blue). The indirect pathway connections are shown in red. The DCN left-right commissural connections are shown in green.*

Figure 4. Animal Setup for MEMRI

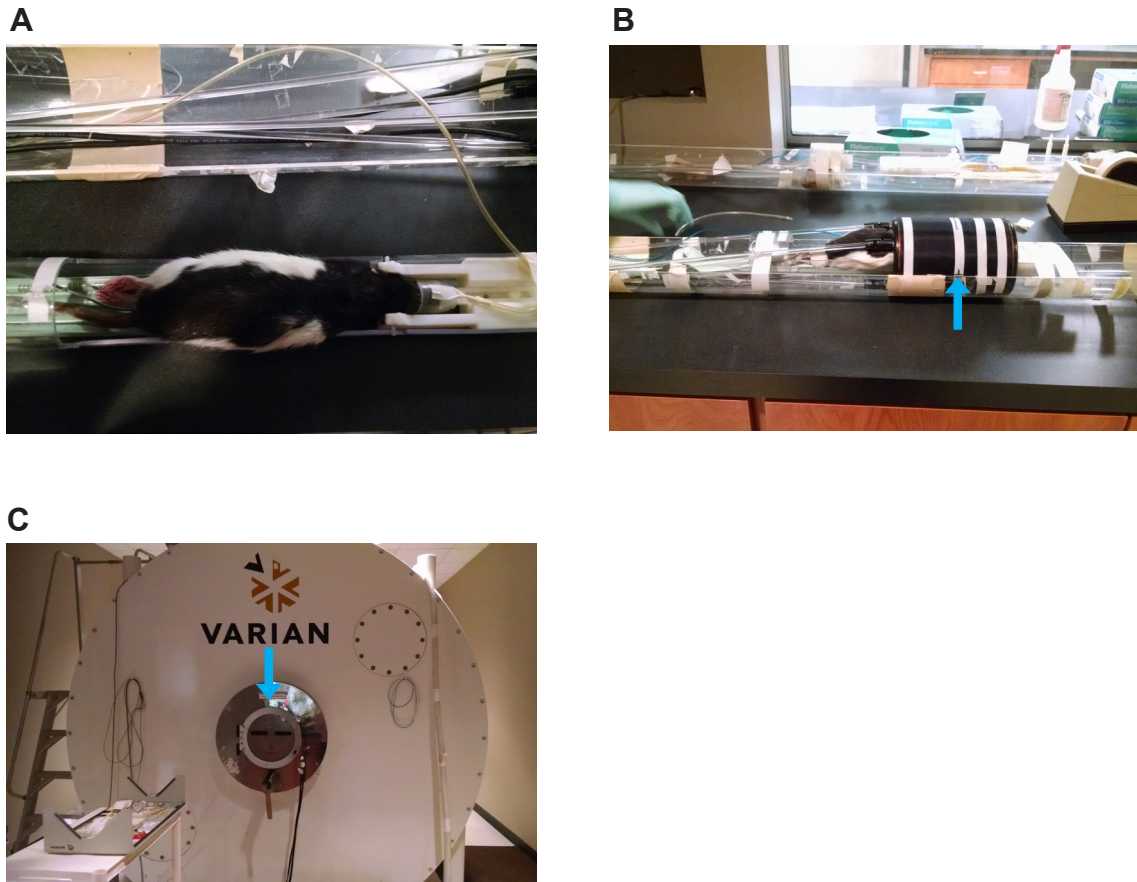


Figure 4. Animal setup prior to brain scan is shown in A and B. Animal subject's (respiration and body temperature) are continuously monitored. Animals were delivered Isoflurane anesthesia via a tube inserted through a nose cone fitted around the animal's nose as shown in A. Ear bars with rubber tips were inserted in each ear to minimize movement of the head. Once animals were set up and physiological readouts were stable, the animal was inserted into the volume coil as shown in B. The arrow indicates the approximate location of the middle of the animals head within the coil; this allowed for optimum signal in each ROI. The 9.4T small animal magnet is shown in C, the cradle containing the animal and coil were inserted into the circular silver opening in the center of the scanner (arrow).

Chapter 2: Sound Damage, Neuroplasticity and Tinnitus Behavior

2.1 Introduction

The most common cause of tinnitus is damage to the peripheral auditory system. Damage induced by overexposure to an acoustic stimulus often results in peripheral deafferentation (Roberts et al 2010). While the original insult may take place in the periphery, sometime later central brain areas may undergo neuroplastic changes (Bauer et al 2013b, Brozoski et al 2007, Dong et al 2010, Engineer et al 2011, Guitton 2012, Kaltenbach et al 2000, Tzounopoulos 2008). Alterations in spontaneous activity, neurotransmission, and protein expression are physiological changes that have been reported in noise and drug induced animal models of tinnitus (Bauer et al 2013b, Brozoski et al 2013, Dong et al 2010, Kraus et al 2010, Sahley et al 2013). The effect of sound damage on genes involved in excitatory and inhibitory neurotransmission revealed unique changes in protein expression that are region specific (Caspary et al 2008, Dehmel et al 2008, Shore et al 2016). mRNA analysis showed down-regulation of inhibitory neurotransmission in the inferior colliculus (Browne et al 2012). Overall excitability in the dorsal cochlear nucleus has been shown to be influenced by both excitatory and inhibitory neurotransmission (Dong et al 2010).

Recently, doublecortin (DCX) protein expression has been evaluated in animal models of tinnitus. Doublecortin (DCX) is a microtubule associated protein expressed exclusively in neuronal tissue. DCX has been used as a marker of migrating and immature neurons (Gleeson et al 1999, Manohar et al 2012). Historically, DCX expression has been correlated with neurogenesis as it is expressed in immature post-mitotic neurons (Francis et al 1999, Friocourt et al 2003, von Bohlen und Halbach 2011). Unipolar brush cells in the auditory cochlear nucleus (DCN) and motor associated cerebellar parafloccular lobe (PFL) have been

shown to express DCX (Manohar et al 2012). Unipolar brush cells are a subpopulation of excitatory glutamatergic interneurons found in the DCN and PFL. Cerebellar unipolar brush cells receive glutamatergic inputs from mossy fibers and form glutamatergic synapses with their target cells (i.e. granule cells and other UBCs). In the DCN, excitatory input from mossy fibers descending from the IC and auditory cortex terminate on the UBCs (Bauer et al 2013b).

The cerebellum has been implicated in both generation and modulation of tinnitus (Bauer et al 2013a, Brozoski et al 2007, Mennink et al 2018) and the PFL has been shown to receive auditory input from the cochlea in chinchilla, cat, and monkey (Rasmussen 1990). The cerebellum also functions as an integrator of somatosensory information from multiple sites (Sawtell 2010, Voogd & Glickstein 1998). In particular, PFL ablation eliminates behavioral evidence of tinnitus in sound damaged rats (Bauer et al 2013a). Application of NMDA antagonists in the PFL has also been shown to modulate tinnitus behavior (Bauer et al 2013b). Additionally, it has been shown that DCX expression is altered in specific brain regions of animals with behavioral evidence of tinnitus (Brozoski et al 2017). These findings suggest that increased DCX expression in this subpopulation of glutamatergic cells may underlie neuroplastic hyperactivity in the central auditory system, including that associated with behavioral evidence of tinnitus.

Sound damage, that may or may not be accompanied by tinnitus, has also been shown to decrease expression of DCX outside of the auditory system in granule cells of the hippocampal dentate gyrus (Kraus et al 2010). Since DCX has been associated with both developmental and adult neurogenesis (Ernst et al 2014, Francis et al 1999, Friocourt et al 2003, Gleeson et al 1999, Klempin et al 2011, Walker et al 2007), and the hippocampus undergoes neurogenesis into adulthood, it was suggested that decreased DCX in the dentate

reflected decreased neurogenesis. However the interpretation of DCX expression in the DCN and PFL is not as clear cut. More studies were necessary to determine if DCX expression in the DCN and PFL meant that perhaps new cells were being generated mitotically in brain regions not thought to undergo neurogenesis past embryonic development. Paolone and colleagues investigated neurogenesis in UBCs using DCX in conjunction with bromodeoxyuridine (BrdU) labeling in the DCN and PFL (Paolone et al 2014). Newly generated cells were tracked by injecting BrdU systemically, followed by immunohistochemistry for both BrdU and DCX. While BrdU labeled cells were apparent in the brainstem and cerebellum, the number and distribution of labeled nuclei do not support the hypothesis of neurogenesis and migration of DCX labeled cells (Paolone et al 2014). Additionally, DCX has a restricted expression pattern and is limited to post-mitotic cells, showing no expression in proliferating cells. DCX has been shown to be localized to the tip of growing neuronal processes where it potentially plays a role in axonal guidance (Friocourt et al 2003). These findings suggest that altered DCX expression may not be associated with the generation of new neurons in the DCN and PFL following sound damage, but rather play a unique role in CNS plasticity in these regions.

To better understand the role of immature neuronal differentiation, migration and neurite outgrowth in tinnitus, we utilized immunohistochemistry to detect DCX protein in the dorsal cochlear nucleus, cerebellar paraflocculus and the dentate gyrus of the hippocampus. DCX expression has been investigated 10 weeks post damage in the hippocampus and ~5 months and 11 months post damage in the DCN and PFL while changes at acute time points have not been investigated. Therefore, examining changes in DCX at an acute time point in three

different brain areas, as proposed here, will provide additional information about the time course of neuroplastic changes in animals with and without tinnitus following sound damage.

2.2 Methods

Animals

Male, Long-Evans rats (Charles River Laboratories, Wilmington, MA) that were 2-3 months old at the time of arrival were used in these experiments. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. All animals had ad libitum access to water and standard laboratory rodent chow. They were housed individually with environmental enrichment and maintained on a 12 hour light-dark cycle. A summary of our experimental timeline is shown in Figure 1.

Auditory Brainstem Response (ABR)

ABRs were recorded for each animal both at baseline and ~ 2 weeks post-damage using Intelligent Hearing Systems Smart EP hardware and software (IHS, Miami, Florida) in a sound attenuated booth (Industrial Acoustics Company, Bronx, NY). Rats were anesthetized with Isoflurane (2-2.5%) delivered via the Matrix VP 3000 isoflurane vaporizer (Midmark, Kettering, OH). Respiration was monitored and body temperature was regulated by a feedback/automatic adjustment heating pad. A probe connected to a high frequency transducer was placed in the left ear and a series of tone bursts was presented at a range of frequencies (2, 4, 8, 11.3, 16, 22.6, and 32 kHz) and intensities. For each frequency, threshold was defined as the lowest intensity (dB SPL) for which a signal could be reliably observed in three or more repetitions. A high pass filter was used for the 22.6 and 32 kHz frequency sweeps to prevent artificially low thresholds at high frequencies. Stimulus presentation started at 70 dB and was decreased in 5-10dB increments until no response was detected. Analysis

of ABR thresholds gave us information about the amount of hearing loss induced by our mild sound damage paradigm.

Gap Detection

Animals were tested for tinnitus behavior using gap detection. Behavioral testing was conducted inside a sound attenuated booth with acoustic startle reflex software and hardware (Kinder Scientific, Poway, CA, see Chapter 1, Figure 1A). Performance was tested using no gap (startle only) and gap trials, with data analyzed to obtain a final gap score that reflected post damage performance compared to baseline for each frequency (Chapter 1, Figure 1B). For the gap detection (GD) procedure each rat was presented with a constant, 60 dB SPL background sound consisting of 1 kHz bands centered at 12, 16, and 20 kHz. Background frequency presentation was intermixed such that 22 trials at each testing frequency (10 gap and 12 no gap trials) were presented in a random order for a total of 66 trials. A 115 dB SPL, 50 ms burst was used to induce the acoustic startle reflex. During the background noise, the startle stimulus was presented alone (no gap trial) or immediately following a silent gap embedded in the background noise (gap trial). Silent gaps were 100 ms in duration with a lead interval of 100 ms relative to the startle stimulus. Animals were tested on 4 different days at baseline and approximately 2 weeks post damage.

For each rat, all startle data (baseline and post damage) were combined into a single spreadsheet and sorted as a function of background frequency, and then sub-sorted by gap/no-gap trial status (Neal 2016). A single iteration of the Grubbs outlier detection test (Grubbs 1950, Longenecker et al 2014) was performed on each subset of data (e.g. 12 kHz gap, 20 kHz no-gap, etc.), removing a maximum of 1 extreme outlier per subset. Outliers were excluded from all further analyses. For each background frequency, force data on a given test

day were averaged to calculate both baseline and post-exposure gap/no-gap ratios. Each animal's post-exposure ratio was normalized using its corresponding baseline ratio (post exposure [gap/no-gap] / baseline [gap/no-gap]), which we refer to as a "Gap Detection Score" or "GD Score." This normalization allows for comparisons of intra-animal changes in performance where a value of 1 indicates no change from baseline, values <1 indicate improvement, and values >1 indicate impairment.

Control data were obtained from 7 animals (no sound damage) to serve as a guide in sorting our animals into tinnitus positive (sound damage with evidence of tinnitus behavior), tinnitus negative (sound damage and no evidence of tinnitus behavior) and improved (sound damage and a significant improvement in GD performance at 2 or more tested frequencies).

Sound Damage

Rats were placed in a sound attenuated booth (Industrial Acoustics Company, Bronx, NY) and anesthetized with Isoflurane (4% Iso, 2 L/min induction, 2% iso, 1.5 L/min O₂ maintenance) administered via the Matrx VP 3000 isoflurane vaporizer (Midmark, Kettering, OH). Respiration was monitored and body temperature was regulated by a feedback/automatic adjustment heating pad. A 16 kHz pure tone was continuously presented to the left ear at 114 dB for 1 hour from a loudspeaker (Radio Shack 40-1310-B) inside a plastic case. The loudspeaker was coupled to the left pinna via ½" flexible plastic tubing and sealed using Audalin ear mold (All American Mold Lab, Oklahoma City, OK). The intensity level of the stimulus measured outside the tubing was 45 dB less than the intensity of the stimulus within the tubing sealed to the head of the animal (Imig & Durham 2005), reducing the likelihood of any bilateral damage resulting from air conduction. A Macintosh computer with a

MaLab synthesizer, event processor, and software (Kaiser Instruments, Irvine, CA) was used to control noise waveform synthesis.

Tissue Preparation

Rats were deeply anesthetized with 5 mg/kg i.p. of Beuthenasia and perfused through the heart with 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS). Brains were removed, divided into two blocks just rostral to the cerebellum in the coronal plane and postfixed in 4% PFA at 4°C, for 24 hours to 1 week. The brain blocks were then cryoprotected in 30% sucrose in PBS for approximately 24 hours or until the brain sank to the bottom of the container. Then the brain blocks were placed in plastic cases, covered with OCT and flash frozen in heptane. Frozen brains were stored at -80°C until further processing.

The brains were then cut into 40µm coronal sections on a cryostat and every section was collected. Sections were stored in 12 well culture plates in a cryoprotectant solution (30% ethylene glycol and 30% glycerol in PBS) at -20°C until further processing.

Immunohistochemistry

All tissue processing was done on free-floating sections in 12 well plates. On the first day of processing, sections were removed from cryoprotectant and rinsed with PBS. Sections were placed in 3% hydrogen peroxide at room temperature (RT) for 5-10 min to quench endogenous peroxidase activity. Sections were rinsed in PBS for 10 min to remove excess hydrogen peroxide. Non-specific binding of primary antibodies was blocked by incubating the sections in a solution of 10% normal horse serum (NHS, Vector Laboratories), containing 0.1% Triton X-100 in PBS for 1 hour at RT. Primary antibody (1:500, Doublecortin- Santa Cruz sc-8066) was diluted in 1% NHS, and 0.1% Triton X-100 in PBS. Sections in the 12 well plates incubated in primary antibody overnight on a rocker at 4°C.

On day 2, sections were removed from the primary antibody solution and rinsed 3x for 10 min each in PBS. Biotintylated secondary antibody (1:300, donkey anti-goat, Vector Laboratories) was diluted in 1% NHS, and 0.1% Triton X-100 in PBS, added to sections and incubated for 1 hour at RT. Sections were then rinsed 3x for 10 min each with PBS. Sections were prepared for antibody visualization using the ABC elite kit according to manufacturer instructions (Vector Laboratories PK-6100). Following a 45-60 minute incubation at RT, sections were rinsed 2x for 5 min each in PBS. Immunoreactivity was visualized using the DAB peroxidase substrate kit with nickel enhancement (Vector Laboratories SK-4100) per manufacturer instructions. Incubation time varied from 5-10min depending on DAB reactivity. Sections were then washed in PBS several times until sections were free of reaction precipitates.

Labeled sections were floated onto slides in PBS and slides were left to dry overnight: Sections then were dehydrated using increasing concentrations of ethanol (70%, 95%, 95%, 100%, 100%), cleared in xylene and cover-slipped with DPX (Millipore Sigma).

Imaging and Immunolabeling Quantification

Immunostained sections were examined with a Nikon 80i bright-field microscope and digital images of specific regions of interest (ROIs) were captured using a Nikon DS-Fi1 High-Definition Color Camera Head and NIS-Elements imaging software. Digital images were collected bilaterally and labeling was measured on 20x (200x total magnification) images stitched to provide a complete rendition of each ROI. Assembly of images for all ROIs was accomplished using a combination of Adobe Illustrator and Adobe Photoshop.

Dorsal cochlear nucleus (DCN):

As shown in Figure 6, the DCN was divided into frequency regions as defined by metabolic mapping (Ryan et al 1988), using Adobe Illustrator and a method modified from Neal (enter his dissertation info 2016). First the dorsal and ventral boundaries of the DCN were drawn and then two additional lines were drawn to divide the DCN into three equal regions, which correspond to low, middle and high frequency quadrants. Illustrator images with divisions then were opened in ImageJ, converted to 8 bit, and frequency region specific boundaries were drawn (Figure 6A). Binary images were created using the Otsu thresholding method (Otsu 1979) and percent area labeled was measured within the entire frequency region. Measurements were made in DCN ipsilateral and contralateral to the damaged ear.

Cerebellar parafloccular lobe (PFL):

DCX labeled images were opened in ImageJ, converted to 8-bit, and the transition zone between the ventral PFL and flocculus was outlined (Manohar et al 2012). Binary images were created using the Otsu thresholding method and percent area labeled was measured bilaterally.

Dentate gyrus of the hippocampus (DG):

DCX labeled images were opened with Adobe Illustrator and the length of the dentate gyrus was measured. Labeled cells in the subgranular zone of the dentate granule cell layer were counted. Cells immunopositive for DCX were counted when the cell body was recognizable and there was at least 1 labeled process extending from the cell body. Labeled cells were clearly darker than the surroundings with cytoplasm homogenously labeled (see

Figure 10). Cell density (number of cells/length of DG) was measured bilaterally in three consecutive sections. Average cell density from all three sections was used in our analysis.

Data Analysis

All statistical tests were carried out using Prism v6.0 (GraphPad, La Jolla, CA) with the level of significance set at $p = 0.05$ for all analyses. Auditory brainstem response threshold changes (post-damage vs. baseline, as a function of frequency) were assessed using repeated-measures Two-way ANOVA, with Fisher's LSD post-hoc test. Doublecortin immunolabeling was assessed using One-way ANOVA, with Sidaks multiple comparisons test (DCN, PFL, DG) and the Mann-Whitney test (PFL and DG)

2.3 Results

Tinnitus Behavior (gap detection)

We want to identify the tinnitus status of individual animals so that we can explore whether doublecortin staining is related to tinnitus. Because we are comparing gap detection performance after sound damage to baseline performance, we included control (unexposed) animals in our experimental design to examine whether gap detection performance changed as a function of testing timeline. Figure 1 shows the tests performed as part of the experimental timeline.

In Figure 2A the gap detection scores of control animals ($n=7$) are plotted as a function of background testing frequency. Mean gap detection scores were near 1 at all frequencies, suggesting no change in performance over time. In Figure 2B the gap detection scores of all sound exposed animals ($n=12$) are plotted superimposed on the range of gap detection scores

from control animals (shaded bars). For sound damaged animals, mean gap detection scores at all 3 frequencies were less than 1, suggesting improvement in performance for the group as a whole. However performance was highly variable among animals, which led us to evaluate individual animals across all frequencies in hopes of identifying subgroups related to tinnitus status. We generated a 'heat map' of each individual animal's performance as shown in Figure 2C. Gap detection scores are compared to mean values for controls at each background frequency. Using these heat maps we identified three subgroups based on their gap detection testing. The tinnitus positive group showed mildly to significantly impaired performance at one or more frequencies (Figure 3B). The tinnitus negative group showed unchanging or mildly improved performance at all frequencies (Figure 3D). Finally, we observed a group that unexpectedly showed significantly improved performance at two or more frequencies (Figure 3F). We will use these three groups (tinnitus positive, tinnitus negative, and improved) for subsequent analyses.

In Figure 3 gap detection scores of individual animals in each group are superimposed onto the range of gap detection scores from control animals (A, C, E) and the heat map (B, D, F) of each individual's performance within that group is shown to the right of each graph. One third of animals showed mild to significant impairment in performance at 16 kHz and 20 kHz. Forty-two percent of animals displayed unchanging to mild improvement in performance at two or more frequencies. Twenty-five percent of animals had significantly improved performance at two or more frequencies.

Auditory Brainstem Response (ABR, hearing loss)

Figure 4A shows the degree of hearing loss that occurred in the ear ipsilateral to the damaging stimulus. When examining all animals, our 114 dB, 1 hour exposure resulted in significantly increased thresholds of hearing at 5 of 7 tested frequencies (8 kHz, $p \leq 0.01$; 11.3, 16, and 22.6 kHz, $p \leq 0.001$; 32 kHz, $p < 0.05$) relative to baseline. Figure 4B shows change in threshold relative to baseline as a function of tinnitus group. We observed no differences in threshold shift among tinnitus groups at any frequency with one exception. There was a significant increase in the magnitude of threshold change in the tinnitus negative group relative to tinnitus positive group at 32 kHz ($p < 0.05$).

Neuroplasticity (immunohistochemistry)

We evaluated doublecortin (DCX) staining in the dorsal cochlear nucleus (DCN) as well as two non-auditory regions (hippocampal dentate gyrus [DG] and cerebellar parafloccular lobe [PFL]). Representative images of DCX labeling in selected areas of interest appear in figures 6, 8 and 10. Regional labeling density was evaluated in DCN (Figures 5, 6, 7) and the cerebellar PFL (Figures 8 & 9), while individual cells were counted in the hippocampal dentate gyrus (Figures 10 & 11). For each region we evaluated labeling for all animals combined as well as for subgroups based on tinnitus status. For statistical analysis, immunoreactivity (IR) was quantified both ipsilateral and contralateral to the damaged ear. This is especially important for the DCN, where input from the eighth nerve is relayed to higher CNS structures in a specific sequence that is dependent on the location of an auditory stimulus (see Chapter 1, Figure 3).

Dorsal cochlear nucleus (DCN): DCX immunoreactivity (IR) was quantified as percent area labeled in high, mid and low frequency regions of the DCN both ipsilateral and contralateral to the damaged ear. Labeling in control animals (Figure 5A) revealed no differences in the ipsilateral DCN label relative to the contralateral side in any frequency region, nor were there differences in density within each side as a function of frequency. In Figure 5B control labeling was compared to labeling in all noise exposed animals. Again there were no ipsilateral or contralateral differences in DCX IR of noise exposed animals relative to controls. Figure 6 provides a qualitative view of DCX IR in the DCN of a representative tinnitus negative animal (B & C) and an improved animal (D & E). These sections were chosen to provide visualization of labeling that corresponds to the two groups in which we saw the biggest differences as shown in Figure 7. When labeling in control animals was compared to each tinnitus groups, as shown in Figure 7A & B, there were no significant differences between controls relative to any tinnitus groups. Significant differences were observed only in the high frequency region of the DCN (HF DCN) (One-Way ANOVA; $F = 9.519$, $p = 0.0004$). More specifically, there was a significant bilateral decrease of DCX IR in the HF DCN of animals in the improved group relative to tinnitus negative animals in this region ($p \leq 0.01$). There was a significant DCX decrease in the contralateral HF DCN of improved animals relative to tinnitus positive animals ($p \leq 0.01$). Lastly, labeling in the ipsilateral HF DCN of tinnitus positive animals was also decreased relative to tinnitus negative animals ($p \leq 0.05$).

Parafloccular lobe (PFL): DCX IR was quantified as percent area labeled in the unipolar brush cell (UBC) rich transition zone between the flocculus and paraflocculus of the cerebellum. Representative images of a control animal (B & C) and a noise exposed animal (D & E) can be seen in Figure 8. In the PFL, unipolar brush cells (UBCs) in the granule cell

layer are densely labeled with DCX. Quantitative analysis (Figure 9A) revealed a significant bilateral increase of DCX labeling when comparing all noise exposed animals relative to controls (One-Way ANOVA $F = 6.551$, $p = 0.0022$). When animals were sorted according to tinnitus status (Figure 9B), there was a significant bilateral increase in DCX labeling of the transition zone of the PFL in tinnitus negative animals relative to controls (Mann-Whitney $p = 0.0173$). The mean for tinnitus positive animals was greater than that for controls but did not reach statistical significance ($p = 0.0952$). PFL sections from two sound damaged animals were damaged during tissue processing thereby we sorted animals into two groups (tinnitus positive and tinnitus negative) rather than three to ensure we had $n > 2$ for each group. Taken together, these results suggest increased PFL labeling is a function of hearing loss rather than tinnitus status.

Dentate Gyrus of the Hippocampus (DG): DCX cell density (number of labeled cells/length of the SGZ) was quantified in the dentate gyrus of the hippocampus. Figure 10 shows a representative section from a control animal and a noise exposed animal in which density appears to be decreased. When DCX cell density was quantified (Figure 11A), there was a significant bilateral decrease in DCX cell density when comparing all noise exposed animals to controls ($F = 22.04$, $p = <0.0001$). As shown in Figure 11B, there was a significant bilateral decrease in all three subgroups (tinnitus positive, tinnitus negative and improved) relative to controls when animals were sorted according to tinnitus status ($F = 9.118$, $p = <0.0001$). Again, given that a significant decrease in DCX is seen in all tinnitus groups, these changes are more likely correlated with hearing loss.

2.4 Discussion

In these experiments we evaluated the relationship between tinnitus and neuroplasticity, as exemplified by DCX label, in both auditory and non-auditory brain regions. Using control animals for comparison, we sorted animals into three groups demonstrating distinct patterns of performance. One third of animals were identified as tinnitus positive and 42 % as tinnitus negative, relative proportions that are typical of sound damage. We also identified a unique group of responders, 25% of the sound exposed group, that showed significant gap detection improvement at 2 or all tested frequencies. We sought to identify whether they also had a unique DCX expression pattern distinct from the other two groups.

Since hearing loss and tinnitus are not mutually exclusive we evaluated whether hearing loss changed as a function of tinnitus status in this study. All sound damaged animals had increased hearing thresholds relative to baseline at five of seven tested frequencies that ranged from 8 kHz to 32 kHz. The only difference among groups was that animals identified as tinnitus negative had more severe hearing loss at 32 kHz relative to tinnitus positive animals. Tinnitus positive animals had almost no change in threshold of hearing at 32 kHz while tinnitus negative animals had a 15 dB increase. Interestingly, hearing profiles of tinnitus positive and improved animals were very similar.

Density of doublecortin (DCX) protein labeling changed as a function of hearing loss and tinnitus status with unique expression changes observed in all three regions of interest investigated here. In the DCN, regional density measurements were similar between controls and sound damaged animals when animals were grouped together. Separating animals into three tinnitus subgroups yielded significant differences that were only present in the high

frequency region of the DCN. However, contrary to other published results reporting increased DCX label in tinnitus animals (Bauer et al 2013a), we observed decreased DCX labeling in the high frequency region of the ipsilateral DCN in tinnitus positive animals relative to tinnitus negative animals. Since this change is present in the DCN ipsilateral to the damaged ear, a decrease in DCX may reflect the mutual decreased glutamatergic input to UBCs two weeks post damage. It may require a longer lasting decrease in eighth nerve input to see increased DCX activity in the DCN as observed by others. Bauer et al. (2013) showed that chronic tinnitus behavior could be reduced by blocking excitatory transmission in UBCs. Thus, knowing when DCX activity changes from decreased (as observed here) to increased (as observed by Bauer et al.) will be an important time point to identify and may allow treatment to be administered prior to tinnitus onset. In the high frequency region of the contralateral DCN we also saw a decrease in the improved group relative to tinnitus positive animals.

Lastly, we identified a bilateral decrease in DCX label in the high frequency region of animals in the improved group relative to tinnitus negative animals. This finding is more challenging to explain as our improved group was identified following the observation of unexpected improvement during gap detection testing. The significant level of improvement we observed may be explained by habituation to the behavioral task, but the pattern of DCX labeling is unique in this group in that DCX density was different in the improved animals relative to both tinnitus negative and tinnitus positive groups. We think it would be interesting to see if we could get a similar group of gap detection performers in another cohort of animals in order to investigate further. These results were different than what we expected and seeing the different profile of expression in improved animals relative to the other two groups strengthens the idea that this small group of animals is unique.

Regional DCX density in the PFL was bilaterally elevated in sound damaged animals relative to controls. When grouped according to tinnitus status we saw a bilateral increase in labeling of the UBC rich region of the PFL in tinnitus negative animals relative to controls. The tinnitus negative group is what appears to be driving the increased DCX label when all animals are grouped together. These results suggest that DCX expression in UBCs and therefore glutamate transmission in the transition zone of the floccular lobe is altered due to sound damage, regardless of tinnitus behavior. This suggests that the plastic changes in the PFL observed in these experiments are likely a result of hearing loss.

Bauer et al. (2013) found that DCX was bilaterally elevated in the DCN and PFL of sound damaged animals when examined 5 to 11 months after damage. A more recent study by Brozoski et al. (2017) did compare DCX labeling among tinnitus groups. This study showed no change in PFL DCX labeling of tinnitus positive animals relative to unexposed and no tinnitus (sound damage without behavioral evidence of tinnitus) animals. However, DCX was decreased in the flocculus (another region within the parafloccular lobe) of animals without tinnitus. Neuronal degeneration was present in both the DCN and PFL of all sound damaged animals regardless of tinnitus status. These seemingly contradictory findings of DCX alterations in animal models of tinnitus may result from the following things: time from damage to sacrifice and labeling, parameters of damaging stimulus, behavior used to identify tinnitus and differing rostral to caudal location of measurement within each ROI. If these differing results in the current study are due to time from damage to labeling then they aid in telling a more complete story of neuroplastic changes resulting from hearing loss that may or may not be related to tinnitus.

The hippocampus is a known site of neurogenesis in the adult brain (Knoth et al 2010) and is known to play a role in memory and emotion (Kraus & Canlon 2012, Kraus et al 2010, Rolando & Taylor 2014). Forty to seventy percent of individuals with tinnitus also suffer from some form of emotional distress (Gomaa et al 2013, Joos et al 2012). Because disorders such as depression and anxiety are comorbid in individuals with tinnitus, it is reasonable to postulate that limbic brain regions may play a role in the onset of tinnitus symptoms. Alternatively, stressors such as sound damage alone could have a more direct impact on the turnover of new cells in the hippocampus. Similar negative effects of stress on hippocampal neurogenesis have been shown in models of chronic pain (Duric & McCarson 2006). Acute stressors and depression behavior have also been correlated with decreased neurogenesis (Gould et al 1992, Snyder et al 2011) while antidepressants have been shown to increase neurogenesis (Miller & Hen 2015). Thus it is important to distinguish between altered DCX expression as a result of sound damage alone or sound damage leading to tinnitus. Kraus et al. (2012) measured changes in DCX labeling 10 weeks after animals were damaged with a more intense stimulus (126 dB, 12 kHz, 2 h) than we used here. We were interested in determining if similar changes were present early on (~2-4 weeks post damage) and how the milder sound damage would compare to one of higher intensity and longer duration. When we grouped all sound damaged animals together we found a significant bilateral decrease in DCX labeling of the hippocampal dentate gyrus. When animals were grouped according to tinnitus status we saw that this decrease was present in all tinnitus groups and all differed significantly from controls. This suggests that the changes we measured at these early time points correlate with hearing loss and not evidence of tinnitus behavior.

If doublecortin expression is in fact a measure of brain plasticity it is clear that its expression is altered differently in the DCN depending on time after damage. Time does not appear to be a factor in changes seen in the PFL, as increased DCX in this brain region has been shown at both early and late time points following damage. However, the results of this study do not provide evidence that DCX changes as a result of tinnitus but rather by sound damage alone. Hippocampal neurogenesis appears to be directly impacted by sound damage, regardless of tinnitus at both early and later time points following damage. It would be valuable to do a set of carefully timed experiments in similar animal cohorts looking at DCX labeling in the same brain areas resulting from the same sound damage paradigm so that a complete, unfragmented timeline from damage to neuroplastic changes to tinnitus onset could be established.

Figure 1. Experimental Timeline



Figure 1. *Experimental timeline used to determine effect of sound damage on doublecortin labeling and tinnitus behavior two weeks post damage. ABR: auditory brainstem response, GD: gap detection, DCX: doublecortin*

Figure 2. Gap Detection Controls and Sound Damaged Animals

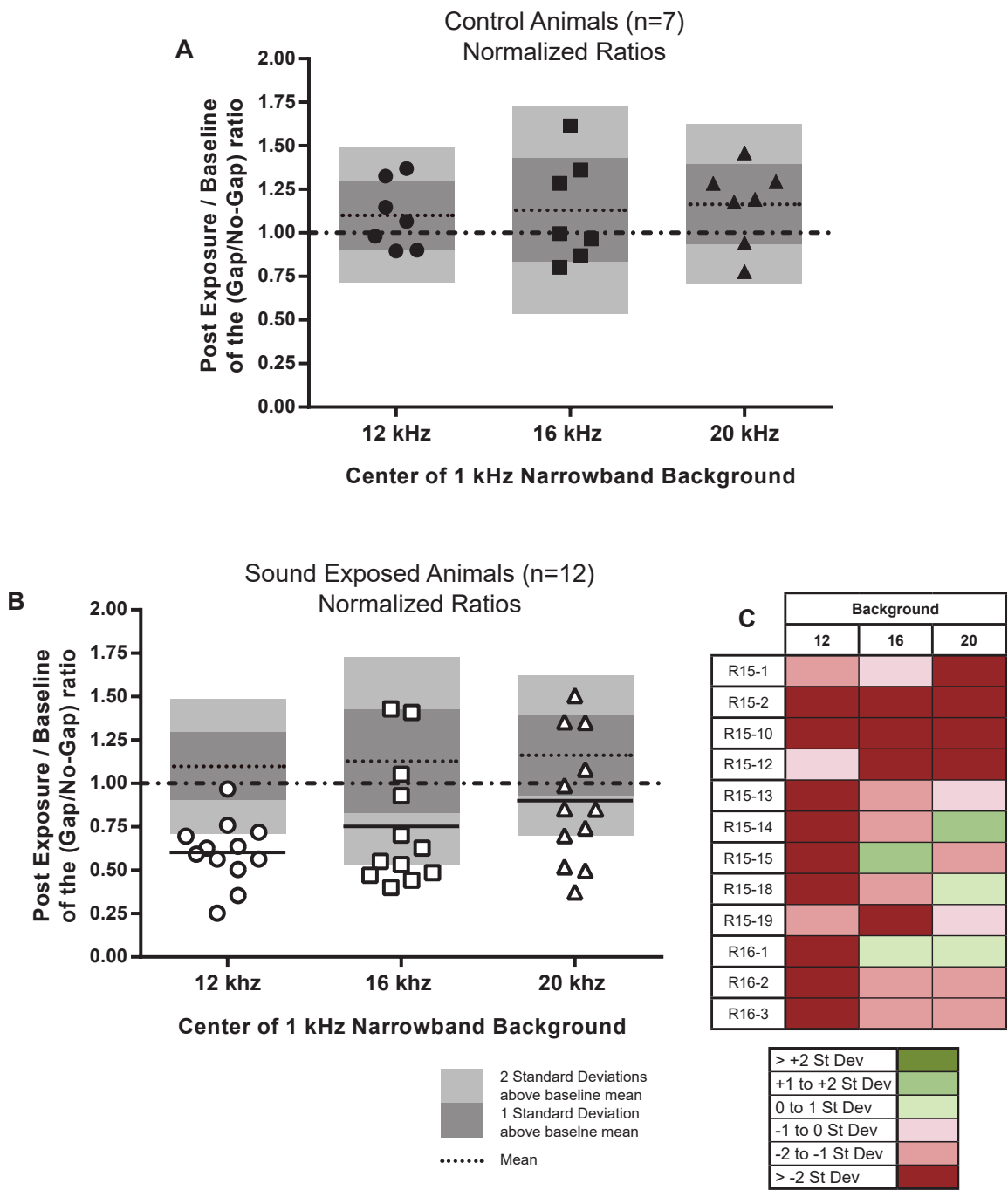


Figure 2. Gap detection data obtained from control ($n = 7$, no sound damage) animals (A). Normalized ratios (post damage/baseline of the (gap/no gap) ratio) for each animal are plotted as function of background noise (12, 16, 20 kHz). The dotted line is the mean of all animals at a given frequency. The shaded gray regions represent standard deviation from the mean (light gray: 2 standard deviations, dark gray: 1 standard deviation). Figure 2B shows gap detection data obtained approximately 2 weeks following sound damage for all animals ($n = 12$). The gap detection score of sound damaged animals is superimposed on the range of values for control animals. For each frequency, dotted line represents the mean of all control animals and solid line represents the mean for all sound damaged animals. A heat map was generated to show each individual animal's performance relative to controls. Dark green to light green was used to indicate impairment in performance relative to controls (GD score $>+2$, -1 or less than 1 SD above controls) and dark red to light red was used to indicate improvement in performance relative to controls (GD score >-2 , -1 , or less than 1 SD below controls). This heat map was used to sort animals into tinnitus groups.

Figure 3. Gap Detection Tinnitus Groups

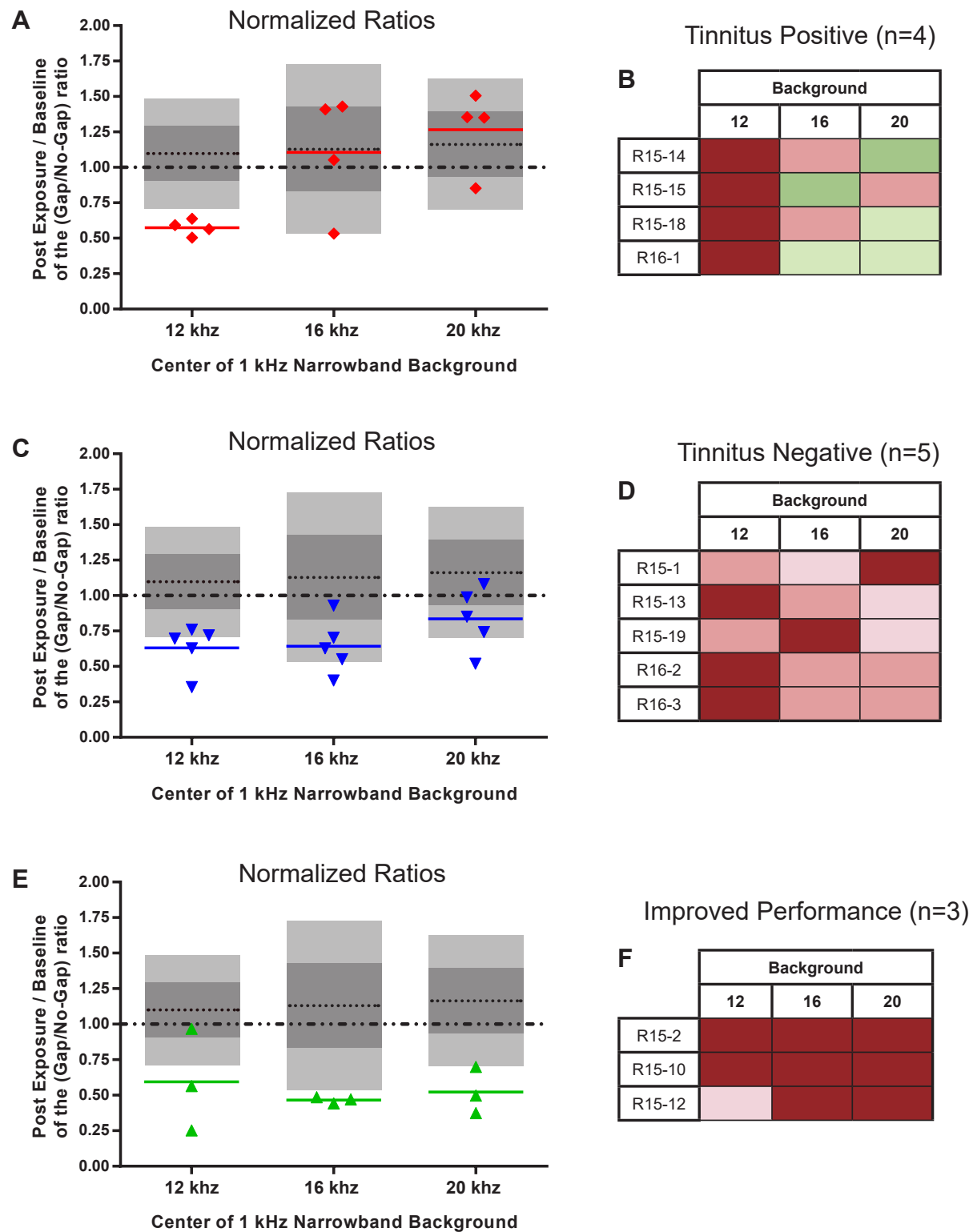


Figure 3. Gap detection data obtained approximately 2 weeks after animals were sound damaged. Data from sound damaged animals was compared to control data to sort animals into tinnitus groups. A, C and E show the performance of sound damaged animals superimposed on the range of values for control animals (shaded gray bars). All other conventions are as in Figure 2. Heat maps (B, D and F) were used to sort animals into tinnitus positive ($n = 4$, impaired performance at one or more frequencies), tinnitus negative ($n = 5$, unchanging or mildly improved performance at all frequencies) and improved performance ($n = 3$, significantly improved performance at two or more frequencies) groups.

Figure 4. Auditory Brainstem Response and Hearing Loss

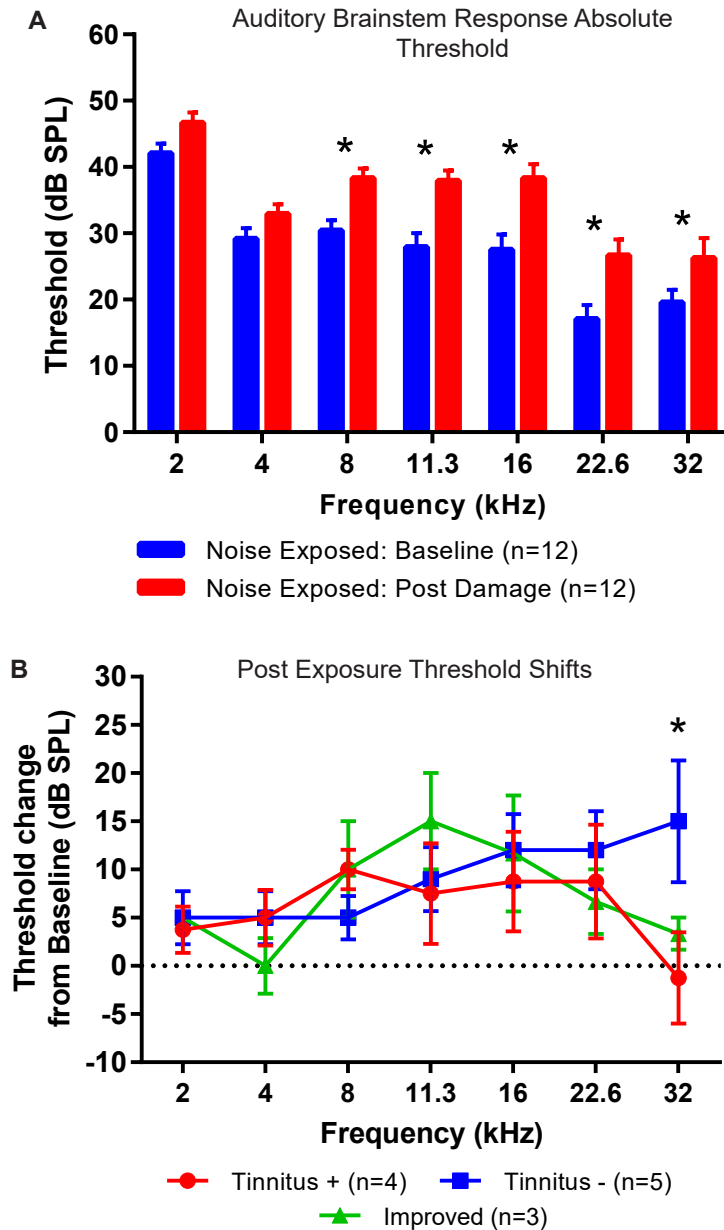


Figure 4. Hearing loss data as evaluated by auditory brainstem response (ABR). The average threshold of hearing at baseline and 2 weeks post damage ($n = 12$) is plotted as a function of frequency (4A). There were statistically significant threshold increases relative to baseline 2 weeks after damage, at 5 of 7 tested frequencies (RM-ANOVA, *indicates $p < 0.05$). 4B compares the change in threshold of hearing for each tinnitus group as a function of frequency. Asterisk indicates the change in threshold of hearing at 32 kHz was significantly greater in the tinnitus negative group ($n = 5$) compared to the other tinnitus groups (tinnitus positive, $n = 4$ and improved, $n = 3$) (One-Way ANOVA, $p < 0.05$).

Figure 5. Frequency Specific Doublecortin Labeling in Controls and Sound Damaged Animals

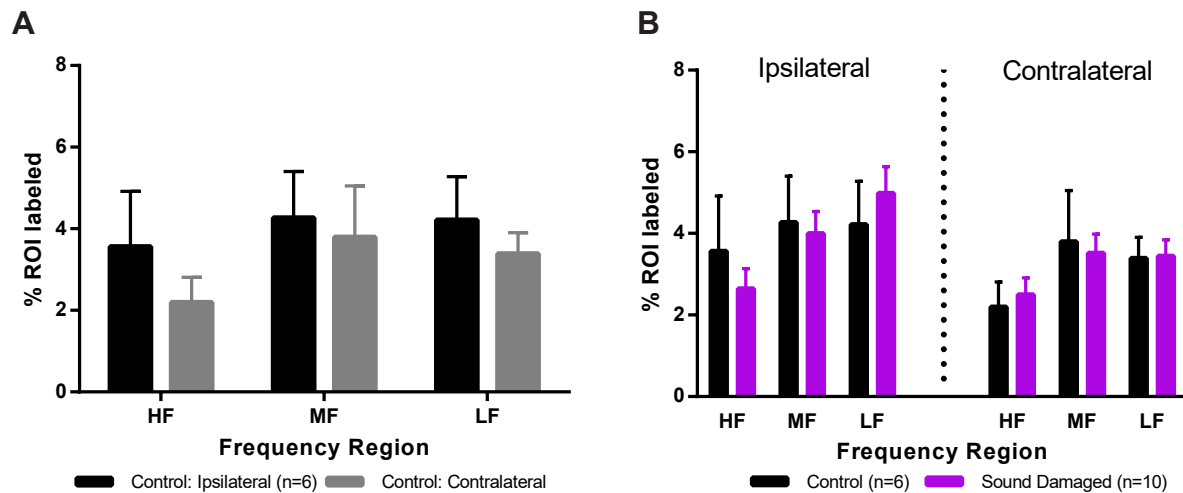


Figure 5. Frequency specific doublecortin (DCX) labeling in the dorsal cochlear nucleus (DCN) of control ($n = 6$) and sound damaged animals ($n = 10$). The DCN was divided tonotopically into high, mid, and low frequency regions (outlined on A in Figure 6) and % ROI labeled with DCX was quantified. 5A shows DCX labeling in control animals (IPSI: Ipsilateral to the damaged ear, CONTRA: Contralateral to the damaged ear). 5B compares ipsilateral and contralateral DCX labeling in control and noise exposed animals as a function of frequency region. HF: high frequency, MF: mid frequency, LF: low frequency, ROI: region of interest.

Figure 6. Doublecortin Immunohistochemical Staining in the Dorsal Cochlear Nucleus: Tinnitus Negative vs. Improved Tinnitus Groups

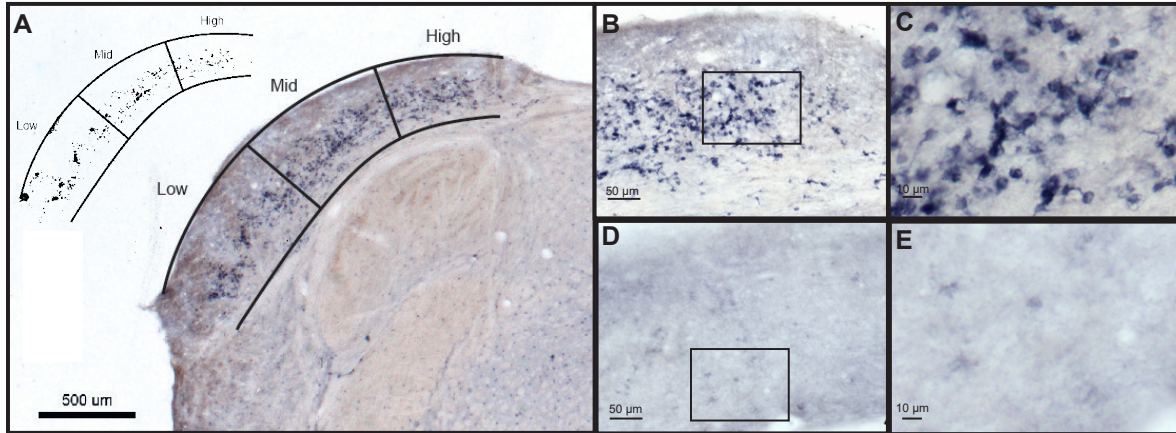


Figure 6. Doublecortin immunohistochemical staining in the dorsal cochlear nucleus: tinnitus negative vs. improved tinnitus groups. A low magnification image of the DCN is shown in A. The DCN was divided tonotopically into high, mid, and low frequency regions (outlined on A) and label was quantified. The inset in A has been thresholded using the Otsu Method in ImageJ and % ROI labeled (black puncta) was quantified in each region. A magnified view of DCX labeling in the high frequency region of a tinnitus negative animal (B) and an animal in the improved group (D) are shown to the right of A. C and E are magnified views of labeled cells contained within the insets shown in B and D.

Figure 7. Frequency Specific Doublecortin Labeling in the DCN by Tinnitus Groups

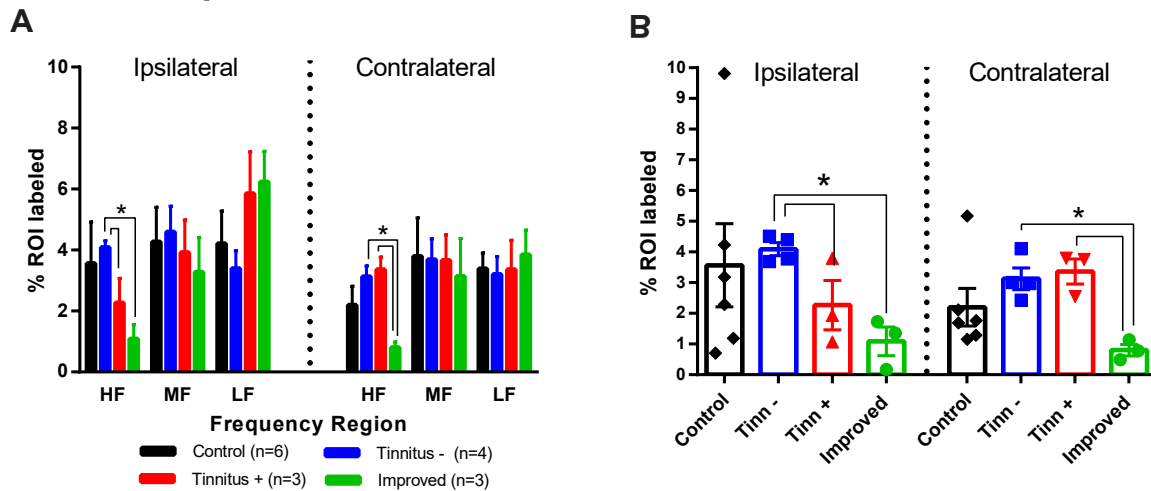


Figure 7. DCN doublecortin labeling compared between control and tinnitus groups. There were no statistically significant differences between controls and any tinnitus group (Two-way ANOVA; $F = 2.027$, $p = 0.12$). Significant differences were observed only in the high frequency region of the DCN (A) (One-Way ANOVA $F = 9.519$, $p = 0.0004$). Scatterplots of high frequency region labeling (7B) show a significant bilateral difference in DCX labeling between tinnitus negative animals ($n = 4$) and animals with improved ($n = 3$) gap detection performance ($p \leq 0.01$). Labeling in the ipsilateral HF region of the DCN of tinnitus positive animals ($n = 3$) was decreased relative to tinnitus negative animals ($p \leq 0.05$). In the contralateral DCN, labeling in tinnitus positive animals was greater than that in animals with improved gap detection performance ($p \leq 0.01$).

Figure 8. Doublecortin Immunohistochemical Staining in transition zone of the Cerebellar Parafoccular Lobe: Controls vs Sound Damaged

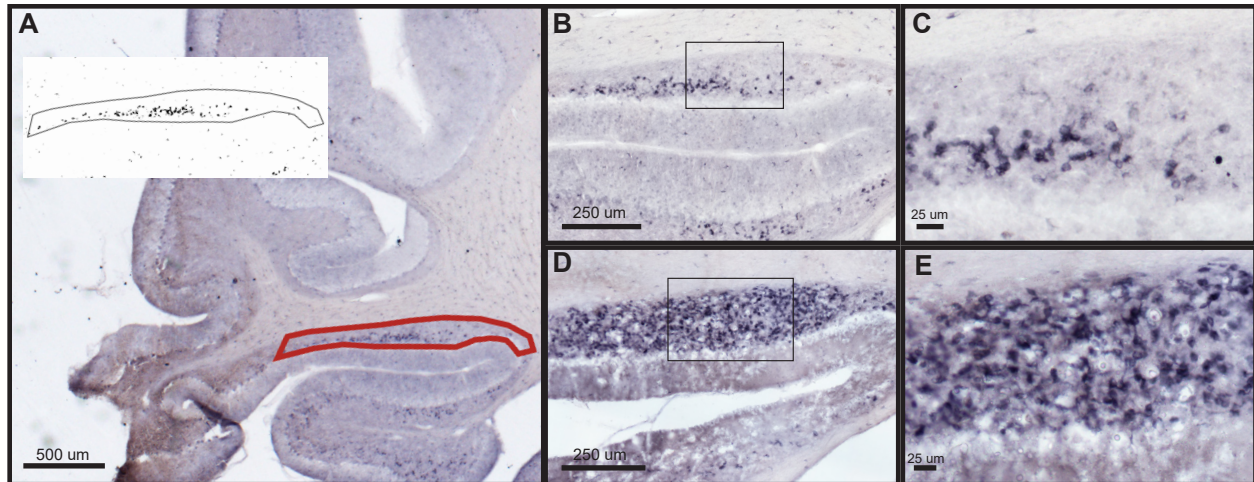


Figure 8. Doublecortin immunohistochemical staining in the transition zone of the cerebellar parafoccular lobe (PFL): control vs. sound damaged. A low magnification image of the cerebellar parafoccular lobe is shown in A. The area outlined in red (the UBC rich transition zone between the parafocculus and flocculus of the cerebellum) was outlined and thresholded using the Otsu Method in ImageJ (inset in A). % ROI labeled (black puncta) in this region was measured bilaterally. A magnified view of DCX labeling in the transition zone of a control animal (B) and tinnitus negative animal (D) are shown to the right of A. C and E are magnified views of labeled cells contained within the insets shown in B and D.

Figure 9. Doublecortin Labeling in the Parafloccular Lobe of Controls and Sound Damaged Animals

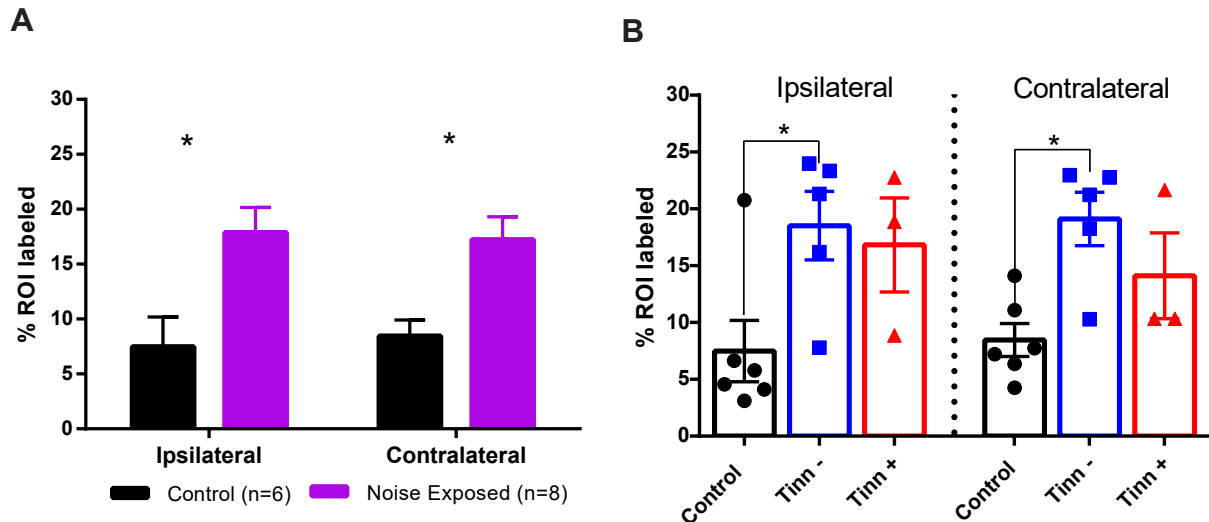


Figure 9. DCX labeling in the PFL of control ($n = 6$) and sound damaged animals ($n = 8$). Data are shown as percentage of ROI labeled in the ipsilateral and contralateral PFL in 9A. Asterisk indicates there was a significant bilateral increase of DCX labeling in the PFL of noise exposed animals relative to controls (One-Way ANOVA $F = 6.551$, $p = 0.0022$). When animals were grouped according to tinnitus assignment (9B), there was a significant bilateral increase of DCX labeling in the PFL of tinnitus negative animals ($n = 5$) relative to controls (Mann-Whitney $p = 0.0173$). Mean DCX labeling in the ipsilateral PFL for tinnitus positive animals ($n = 3$) was greater than that for controls but did not reach statistical significance (Mann-Whitney $p = 0.0952$). Due to tissue damage during processing in two animals, note the change in number of sound damaged animals from $n = 10$ noted in Figure 5 to $n = 8$ in Figure 9. As a result of these decreased numbers, animals in this figure were grouped into tinnitus negative and tinnitus positive only to ensure $n > 2$ for each group.

Figure 10. Doublecortin Immunohistochemical Staining in the Hippocampal Dentate Gyrus: Control vs. Sound Damaged

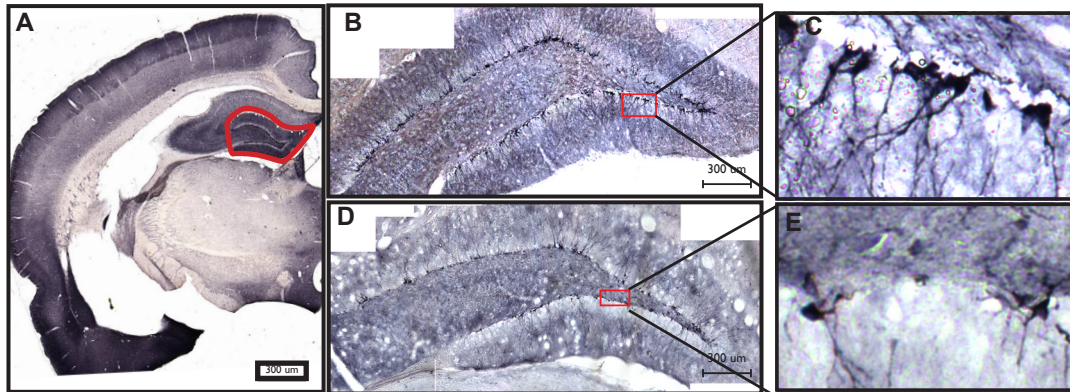


Figure 10. *Doublecortin immunohistochemical staining in the dentate gyrus of the hippocampus in control and sound damaged animals. 10A shows the rostral to caudal level of the hippocampus at which the dentate gyrus (red outline) was sampled. A magnified view of the left dentate gyrus from representative control (B & C) and sound damaged (D & E) animals demonstrates staining intensity. Cells labeled with DCX can be seen to the right of B and C (red outlined boxes).*

Figure 11. Doublecortin Labeling in the Hippocampal Dentate Gyrus of Controls and Sound Damaged Animals

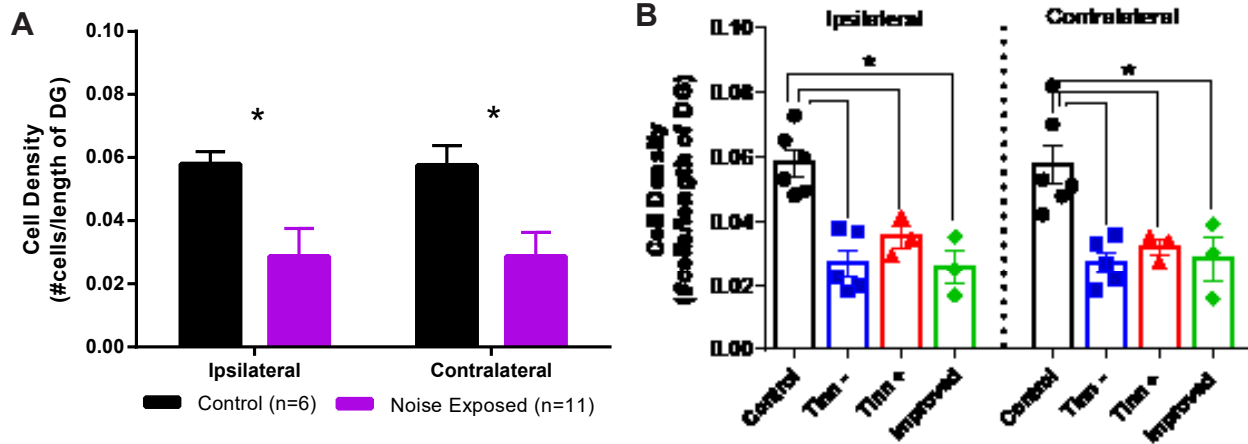


Figure 11. Doublecortin labeling in the dentate gyrus (DG) of the hippocampus of control ($n = 6$) and sound damaged animals ($n = 11$). A bilateral decrease in density of labeled cells (number of labeled cells/length of the DG) was observed in sound damaged animals relative to controls (One-Way ANOVA $F = 22.04$, $p < 0.0001$ as shown in A. When grouped by tinnitus status, there was a significant bilateral decrease in density of labeled cells in tinnitus negative ($n = 5$, $p = 0.0043$), tinnitus positive ($n = 3$, $p = 0.0238$) and improved performance animals ($n = 3$, $p = 0.0238$) relative to controls (Mann-Whitney).

Chapter 3: Sound Damage, Spontaneous Brain Activity, Tinnitus Behavior and Anxiety Behavior

3.1 Introduction

The onset of tinnitus following a peripheral insult is often attributed to aberrant changes in spontaneous activity in auditory brain regions (Dong et al 2010, Kaltenbach 2011, Kaltenbach et al 2002, Seki & Eggermont 2003). Sound damage produces hair cell loss that subsequently leads to hearing loss and loss of input from the periphery. Aberrant input from the periphery eventually results in dysregulation of inhibitory and excitatory transmission throughout the auditory system (Bauer et al 2013b, Brozoski et al 2013, Wang et al 2011, Wang et al 2009). While changes in spontaneous activity have been demonstrated at single time points in many animal models of tinnitus, much less is known about how spontaneous activity changes over time following tinnitus induction. Work done by Guitton (Guitton & Dudai 2007) suggests that there is a brief consolidation window during which permanent damage may be prevented.

In addition to the anticipated auditory brain regions impacted by tinnitus, human and animal studies have shown that non-auditory brain regions are also affected in some individuals. Tinnitus studies utilizing various neuroimaging techniques have shown that limbic regions (Kraus & Canlon 2012, Leaver et al 2011) may exhibit altered activity when tinnitus is present. The hippocampus and amygdala, limbic brain regions, interact with the central auditory system (Rauschecker et al 2010). The basolateral nucleus of the amygdala is involved in evaluation of auditory stimuli which suggests that stressors such as prolonged, intense noise could impact amygdala function (Balleine & Killcross 2006). The hippocampus

also responds to sound stimuli through both direct and indirect input from auditory association cortices via forebrain pathways such as the amygdala. The main function of hippocampus-auditory interactions is the formation of long-term auditory memories (Kraus & Canlon 2012). Rauschecker et al. (2010) suggest that limbic brain regions may be involved in blocking the tinnitus percept from reaching the auditory cortex. When this mechanism is compromised, chronic tinnitus results (Rauschecker et al 2010). It has been reported that in about 70 percent of individuals with tinnitus (Gomaa et al 2013, Holmes & Padgham 2011, Shargorodsky et al 2010), comorbid anxiety and/or depression may be present. This affective component of tinnitus has been less frequently studied and it's unclear whether the impact of tinnitus on quality of life leads to symptoms of anxiety and/or depression, or if mood disorders occur due to direct CNS changes to limbic brain regions as a result of tinnitus. More recently, studies have shown that the cerebellar paraflocculus (PFL) may play a vital role in tinnitus generation. Bauer et al. (2013a) and Brozoski et al. (2017) have found altered protein expression in a subset of glutamatergic cells located in the PFL of animals with behavioral evidence of tinnitus.

Manganese enhanced MRI (MEMRI) is an innovative imaging tool that has recently been used to study neuronal brain activity in animal models of tinnitus (Brozoski et al 2007, Brozoski et al 2013, Cacace et al 2014, Groschel et al 2011, Silva & Bock 2008). Table 1 highlights the details of past studies utilizing MEMRI to measure brain changes that result from auditory insult and correlate with the onset of tinnitus symptoms. Mn^{2+} is a calcium analog that enters active cells via voltage gated calcium channels (Brozoski et al 2007, Eschenko et al 2010, Holt et al 2010). Following systemic $MnCl_2$ injection, Mn^{2+} is taken up and retained within active cells. Mn^{2+} subsequently slowly diffuses out of activated cells, thereby allowing evaluation of labeling patterns for a substantial period of time (e.g. 24 hours) after the stimulus

(Silva et al 2004). This feature of Mn^{2+} uptake prior to MRI is important in auditory research. The noise produced by the scanner can result in auditory stimulation, potentially confounding experiments designed to evaluate brain activity under a specific auditory condition. $MnCl_2$ does eventually diffuse out of cells, so that MEMRI can be employed as a non-invasive, repeatable way to measure whole brain activity. Here, we evaluate activity changes at acute (1 months post sound damage) and chronic (3.5 months post sound damage) time points. An additional benefit of MEMRI is that $MnCl_2$ uptake can be evaluated in unanesthetized animals, making the contrast label indicative of brain activity not influenced by anesthesia. Effects of anesthesia on auditory neuronal activity as measured by electrophysiology have been shown in animals (Anderson & Young 2004, Gaese & Ostwald 2001, Kennon-McGill 2014, Kuwada et al 1989).

In the clinical population, hearing loss does not always result in tinnitus (Konig et al 2006). Conversely, tinnitus can be present in an individual with no audiometric evidence of hearing loss (Martines et al 2010). In animal studies, a sound damaging stimulus that results in hearing loss is often used to induce tinnitus. However, in animals as in humans, sound damage-associated hearing loss does not always result in behavioral evidence of tinnitus (Engineer et al 2011, Kraus et al 2010, Longenecker & Galazyuk 2012, Pace & Zhang 2013). Thus, it is important to employ a behavioral measure of tinnitus, allowing comparison of tinnitus positive and tinnitus negative animals.

A modified version of the acoustic startle reflex is commonly employed in animals to test for tinnitus behavior (Eggermont 2013, Holt et al 2010, Lobarinas et al 2013b, Longenecker & Galazyuk 2012, Pace & Zhang 2013, Turner & Parrish 2008). In this study, gap detection performance was used as described by Neal (2016) to determine if sound damaged animals

displayed behavioral evidence of tinnitus (Neal 2016). This behavioral test does not require animal training; therefore tinnitus assessment can be done over a short period of time. Measured gap detection performance is based on the assumption that an animal's tinnitus fills in a silent gap presented in a background of constant noise.

In these experiments we exposed young adult rats to intense sound to induce hearing loss and tinnitus. We used non-invasive manganese-enhanced MRI at two time points to evaluate changes in spontaneous activity in three auditory and three non-auditory brain regions. We also measured hearing loss, tinnitus behavior and anxiety behavior in each animal at both time points, allowing us to evaluate relationships between hearing loss, tinnitus and brain activity in auditory and non-auditory brain regions.

3.2 Methods

Animals

Male, Long-Evans rats (Charles River Laboratories, Wilmington, MA) that were 2-3 months old at the time of arrival were used in these experiments. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. All animals had ad libitum access to water and standard laboratory rodent chow. They were housed individually with environmental enrichment and maintained on a 12 hour light-dark cycle. A summary of our experimental timeline is shown in Figure 1. Note that animal numbers change throughout the course of our experiments due to animal deaths between the one month and three and a half month time point measurements.

Auditory Brainstem Response

Baseline and post-exposure (1month and 3.5 months after damage) ABRs were recorded for each animal using Intelligent Hearing Systems Smart EP hardware and software (IHS, Miami, Florida) in a sound attenuated booth (Industrial Acoustics Company, Bronx, NY). Rats were anesthetized with Isoflurane (2-2.5%) delivered via the Matrx VP 3000 isoflurane vaporizer (Midmark, Kettering, OH). Respiration was monitored and body temperature was regulated by a feedback/automatic adjustment heating pad. A probe connected to a high frequency transducer was placed in the left ear and a series of tone bursts was presented at a range of frequencies (2, 4, 8, 11.3, 16, 22.6, and 32 kHz) and intensities. For each frequency, threshold was defined as the lowest intensity (dB SPL) for which a signal could be reliably observed in three or more repetitions. A high pass filter was used for the 22.6 and 32 kHz frequency sweeps to prevent artificially low thresholds at high frequencies. Stimulus presentation started at 70 dB and was decreased in 5-10dB increments until no response was detected. Analysis of ABR thresholds gave us information about the amount of hearing loss induced by our mild sound damage paradigm.

Gap Detection

Animals were tested for tinnitus behavior using gap detection. Behavioral testing was conducted inside a sound attenuated booth with acoustic startle reflex software and hardware (Kinder Scientific, Poway, CA, see Chapter 1, Figure 1A). Performance was tested using no gap (startle only) and gap trials, with data analyzed to obtain a final gap score that reflected post damage performance compared to baseline for each frequency (Chapter 1, Figure 1B). For the gap detection (GD) procedure each rat was presented with a constant, 60 dB SPL background sound consisting of 1 kHz bands centered at 12, 16, and 20 kHz. Background

frequency presentation was intermixed such that 22 trials at each testing frequency (10 gap and 12 no gap trials) were presented in a random order for a total of 66 trials. A 115 dB SPL, 50 ms burst was used to induce the acoustic startle reflex. During the background noise, the startle stimulus was presented alone (no gap trial) or immediately following a silent gap embedded in the background noise (gap trial). Silent gaps were 100 ms in duration with a lead interval of 100 ms relative to the startle stimulus. Animals were tested on 4 different days at baseline, 1 month post damage and 3.5 months post damage. For each background frequency, a gap score (post damage gap/no gap ratio)/(baseline gap/no gap ratio) was calculated each day and an average of these 4 scores was calculated to obtain the final gap score for each animal.

For each rat, all startle data (baseline and post damage) were combined into a single spreadsheet and sorted as a function of background frequency, and then sub-sorted by gap/no-gap trial status (Neal et al., 2018). A single iteration of the Grubbs outlier detection test (Grubbs, 1950; Longenecker et al., 2014) was performed on each subset of data (e.g. 12 kHz gap, 20 kHz no-gap, etc), removing a maximum of 1 extreme outlier per subset. Outliers were excluded from all further analyses. For each background frequency, force data on a given test day were averaged to calculate both baseline and post-exposure gap/no-gap ratios. Each animal's post-exposure ratio was normalized using its corresponding baseline ratio (post exposure [gap/no-gap] / baseline [gap/no-gap]), which we refer to as a "Gap Detection Score" or "GD Score." This normalization allows for comparisons of intra-animal changes in performance where a value of 1 indicates no change from baseline, values <1 indicate improvement, and values >1 indicate impairment. Control data were obtained from 7 animals (no sound exposure) to serve as a guide in sorting our animals into tinnitus positive (sound

damage with evidence of tinnitus behavior) and tinnitus negative (sound damage and no evidence of tinnitus behavior).

Open Field Test

The Force Plate Actimeter (BASi, West Lafayette, IN) was used to measure animal location during exploratory behavior within an open field, with less time spent in central areas an indicator of anxiety. The actimeter arena consists of a 356 mm square, stiff horizontal plate attached at the corners to force transducers. A Plexiglas enclosure rests a few millimeters above the plate to create a transparent enclosure. Animals were brought to the testing room and allowed to acclimate for at least 30 minutes prior to experimentation. Immediately prior to each use, the actimeter was wiped with 70% ethanol. Animals were placed into the testing arena and allowed to move freely for 5 minutes. During this time, the actimeter recorded several parameters such as total distance traveled, area traveled, time spent in the center (200 mm x 200 mm), time spent in perimeter (100 mm strip around the perimeter of the arena). An example of the behavioral readout collected during testing can be seen in Chapter 1, Figure 2. Time spent in the center and perimeter was exported from testing software to Excel for further analysis. Percentage of time spent in the perimeter was calculated ($\% \text{ perimeter} = \text{time spent in perimeter (s)} / \text{total time (s)} * 100$) and plotted as function of time point. Difference scores from baseline for 1 month and 3.5 months post damage to baseline were calculated and z-scores were generated and used for correlation analysis.

Sound Damage

Rats were placed in a sound attenuated booth (Industrial Acoustics Company, Bronx, NY) and anesthetized with Isoflurane (4% Iso, 2 L/min O₂ induction, 2% iso, 1.5 L/min O₂

maintenance) administered via the Matrix VP 3000 isoflurane vaporizer (Midmark, Kettering, OH). Respiration was monitored and body temperature was regulated by a feedback/automatic adjustment heating pad. A 16 kHz pure tone was continuously presented to the left ear at 114 dB for 1 hour from a loudspeaker (Radio Shack 40-1310-B) inside a plastic case. The loudspeaker was coupled to the left pinna via ½" flexible plastic tubing and sealed using Audalin ear mold (All American Mold Lab, Oklahoma City, OK). The intensity level of the stimulus measured outside the tubing was 45 dB less than the intensity of the stimulus within the tubing sealed to the head of the animal (Imig et al., 2005), reducing the likelihood of any bilateral damage resulting from air conduction. A Macintosh computer with a MaLab synthesizer, event processor, and software (Kaiser Instruments, Irvine, CA) was used to control noise waveform synthesis.

Manganese-enhanced MRI

Twenty-four hours prior to scan, animals were weighed and given an injection of 66 mg/kg (i.p.) of MnCl_2 (Holt et al., 2010). The MnCl_2 solution was prepared as described previously (Silva et al., 2004 and Rodriguez et al., 2015). Briefly, 100mM of highly purified $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (product number 529680; Sigma-Aldrich) was dissolved in 100 mM Bicine solution and adjusted to pH 7.4. Animals were housed singly in their home cages in a sound attenuated booth for a 24 hour uptake period prior to scanning. The choice of a 24 hour uptake period was based on work previously published indicating that 24 hours post injection allows for optimal enhancement as detected by MRI (Groschel et al 2011). Utilizing this approach allowed for reporting on brain activity during the uptake period and prior to the scan while the animals were awake and freely moving. Therefore, the noisy scanning environment

and anesthetic had very little opportunity to influence results. After 24 hours, animals were transported to Hoglund Brain Imaging Center for the MRI.

Image Acquisition

Twenty-four hours post MnCl_2 injection, animals were anesthetized with Isoflurane (induction: 4% ISO-air flow 2 L/min-oxygen flow 2 L/min, maintenance: 2-3% ISO-air flow 1.5 L/min-oxygen flow .75 L/min) administered via the Matrx VP 3000 isoflurane vaporizer (Midmark, Kettering, OH). For the duration of the scan rats were on a heated re-circulating water pad with a rectal thermometer to monitor and maintain core body temperature. A respiration sensor (Small Animal Monitoring & Gating System, SA Instruments, Stony Brook, New York, USA) was placed underneath the animal in a position that allowed us to monitor the animal's respiration. A custom cradle was engineered to provide stereotaxic control of the animal's head and to achieve optimum positioning inside the coil. A bite bar and rubber tipped ear bars were utilized to stabilize the animal and minimize any movement during imaging. Chapter 1, Figure 4 shows an animal situated in the cradle prior to MEMRI scan as well as the small animal magnet used for imaging.

Manganese-enhanced MRI scans were performed on a 9.4T Varian, now Agilent, small animal scanner (Agilent Technologies, Santa Clara, CA) using a transmit/receive volume coil with a 6.3 cm inner diameter. At each imaging time point (baseline, 1 month post damage and 3.5 months post damage) we ran three different scan sequences to obtain an anatomical image, T1 map and B1 map to use in our analysis. Eleven coronal slices with a slice thickness of 2 mm were obtained with each sequence. Data acquisition and image processing were conducted using VNMRJ software version 1.1 revision D (Varian Inc., Palo Alto, CA).

A Spin Echo Multi Slice sequence was used to acquire high resolution anatomical images for identifying regions of interest (repetition time [TR] 500 ms; echo time [TE] 13 ms; number of averages [NT] 4; matrix size 256 x 256; field of view [FOV] 3 x 3 cm²; providing a resolution of 117 µm x 117 µm; acquisition time=8 minutes 44 seconds). The images were then downsampled to a matrix size of 128 x 128 to match the matrix size of our T1 and B1 maps.

A T1 map for measuring manganese contrast was generated using a non-echo planar, multi-slice T1-based sequence pulse ([TR] 4 ms; [TE] 2 ms; [NA] ; matrix size 128 x 128; [FOV] 3 x 3 cm²; providing a resolution of 234 µm x 234 µm; flip angle=20°; 22 inversion times from 40–5470 ms; acquisition time=8.5 min) The sequence was modified from the Look-Locker sequence (Look & Locker 1970) to acquire multiple slices during one inversion; a total of 22 inversions times (equally spaced from 36 ms to 5244 ms) were acquired. T1 relaxation values were estimated using IDL (Interactive Data Language, Exelis Inc, Boulder, CO) from a 3-parameter fit to an inversion recovery equation.

Finally, a B1 map was generated to correct the effect of flip angle variations on T1 values. The B1 sequence consisted of a two-excitation, same flip angle gradient echo sequence (TR = 200 ms; TE = 2.6 ms; matrix size = 128 ×128; FOV = 3 x 3 cm²; providing a resolution of 234 µm 234 µm; flip angle = 30 degrees; acquisition time 1 minute 51 seconds) (Pan et al 1998). B1 values were calculated in IDL.

MEMRI Image Analysis

High-resolution anatomical images were used to choose slices from each scan series that best represented auditory and limbic brain regions. Digital images from the Paxinos and Watson rat brain atlas (6th edition, 2007) were overlaid on the anatomical images and used to

systematically define regions of interest (ROIs). Auditory areas included the: inferior colliculus (IC), dorsal cochlear nucleus (DCN), and primary auditory cortex (ACtx). Non-auditory areas included the: parafloccular lobe of cerebellum (PFL), hippocampus (HIP), and basolateral amygdala (BLA). An optimum digital atlas image was chosen for each ROI by utilizing recognizable anatomical features of each image and aligning the atlas overlay with the MRI image containing each ROI. The MRI images were scaled up in size and 1 mm grid lines were drawn on the images in Photoshop to allow for more accurate alignment of the two images. ROIs were outlined on the overlaid image and saved in ImageJ (Schneider et al 2012). Mean B1 and T1 values were calculated directly from the T1 and B1 maps utilizing the previously drawn ROIs. Within each ROI, each T1 relaxation value and the corresponding B1 value were manually checked to insure proper parameter fit and T1 value estimate. Pixels corresponding to badly fitted T1 relaxation values were omitted. Additionally, the Grubbs Outlier Test was utilized to remove one outlier from each data set.

R1 ($1/T1$) corrected values, which represent regional manganese signal intensity, were calculated from T1 relaxation values and B1 values according to Kim et al., 2011. To allow for functional comparisons of MEMRI data, R1 values for each ROI were normalized to the R1 corrected value of adjacent muscle tissue (normalized R1 value = R1 value of ROI/R1 value of muscle) as reported elsewhere (Holt et al 2010). Normalization with muscle tissue compensates for signal intensity gradients that remain after image processing in addition to inter- individual and injection time point differences in peripheral $MnCl_2$ uptake (e.g. liver sequestration). For each ROI, the Wilcoxon Signed-Rank Test was used to compare signal intensity measured at baseline to signal intensity measured at 1 month and 3.5 months post

damage. Each animal's own baseline scan served as a control enabling us to monitor individual animals over time.

Data Analysis

All statistical tests were carried out using Prism v6.0 (GraphPad, La Jolla, CA) with the level of significance set at $p = 0.05$ for all analyses. Auditory brainstem response threshold changes (post-damage vs. baseline, as a function of frequency) were assessed using repeated-measures Two-way ANOVA, with Fisher's LSD post-hoc test. Signal intensity as measured using MEMRI was assessed using the Wilcoxon Signed-Rank test.

As part of our experimental design we evaluated changes in multiple dependent variables (e.g. hearing loss, tinnitus and anxiety behavior, brain activity) in every animal at baseline and after sound damage. This allowed us to assess correlations among variables that may reveal significant effects of sound damage. For this analysis, data obtained from all measures were standardized by calculating Z scores. The mean and standard deviation used to calculate each Z score are based on change scores that take into account baseline and post damage measurements (ABR, post damage-baseline; GD, post damage gap ratio/baseline gap ratio; MEMRI, post damage-baseline; OFT, post damage-baseline). To assess these relationships we used Pearson Correlation and Linear Regression analysis with significance set at $p = 0.05$.

3.3 Results

Gap Detection (tinnitus behavior)

We identified the tinnitus status of each individual animal to explore whether hearing loss and density of manganese uptake in specific auditory and non-auditory brain regions are

related to tinnitus at 1 month and 3.5 months post damage. Animals underwent manganese uptake in a quiet environment, so manganese distribution should reflect spontaneous activity.

Because our gap detection score is the ratio of performance post damage to that at baseline for each animal, we included control (unexposed) animals in our experimental design to examine whether gap detection performance changed solely as a function of our testing timeline. We collected behavioral data from control unexposed animals at 1 month after their initial baseline testing. In Figure 2 the gap detection scores of control animals (n=7) are plotted as a function of testing frequency. Mean gap detection scores were near 1 at all frequencies, suggesting little to no change in performance over time. Gap detection scores for damaged animals were compared to those from controls in subsequent analyses.

In Figure 3A-F the gap detection scores of all sound damaged animals 1 month post damage (n=10) are plotted superimposed on the range of gap detection scores from control animals (shaded bars). When all sound damaged animals were grouped together (Figure 3A), mean gap detection scores at 16 kHz and 20 kHz were very similar to the control means suggesting no change in performance. At 12 kHz, the performance of the sound damaged animals improved significantly as a group. Gap detection performance was highly variable among animals, which led us to evaluate individual animals across all frequencies in hopes of identifying subgroups related to tinnitus. We generated a heat map of each individual animal's performance as shown in Figure 3B (all sound exposed animals, n=10). Gap detection scores were compared to mean values for controls at each background frequency. Using these heat maps we identified two subgroups based on their gap detection testing. Animals were identified as tinnitus positive (n=6) if they showed at least mildly impaired performance at one or more tested frequencies (Figure 3C-D). In this cohort, impaired performance occurred at

either 16 or 20 kHz; all animals showed significant improvement in performance at 12 kHz. Animals were identified as tinnitus negative (n=4) if they showed improved performance at all tested frequencies (Figure 3E-F).

Figure 4A-F shows gap detection data 3.5 months post sound exposure. When all sound exposed animals (Figure 4A, n=7) were grouped together, mean gap detection scores were similar to controls when tested at 16 kHz and 20 kHz. Yet again we see a significant improvement in performance at 12 kHz. Animals were identified as tinnitus positive (Figure 4C-D, n=3) if they displayed significantly impaired performance compared to controls at one or more tested frequencies. Animals were identified as tinnitus negative (Figure 4E-F, n=4) if they had improved performance at all tested frequencies.

Our control data were collected from unexposed animals after only the 1 month wait period; we did not re-evaluate GD scores at 3.5 months. Therefore, we took an extra step when assigning animals to a tinnitus group at 3.5 months post damage. Figure 5 plots gap detection scores for individual animal's performance as a function of time after damage. This figure provides further support for our method of tinnitus grouping. At 3.5 months post damage we identified the same 3 animals as tinnitus positive (based now on a GD score >1 at one frequency) and the same 4 animals as tinnitus negative (GD score of <1 at all three frequencies). It's important to note that an animal's tinnitus grouping did not always hold constant over time. Tinnitus status remained the same as it was at 1 month for only three of the seven animals tested at 3.5 months. For 4 of 7 animals (63%) tinnitus status changed over time. Unfortunately, due to small sample size, we cannot look further at these animals to examine how maintenance or changes in tinnitus status affects changes in MEMRI signal intensity.

Auditory brainstem response (ABR, hearing loss)

We utilized the auditory brainstem response to measure changes in threshold of hearing at each time point (1 month and 3.5 months post damage) relative to baseline. Figure 6A shows the degree of hearing loss ipsilateral to the damaged ear that resulted from exposure to a 114dB, 16 kHz pure tone for the duration of 1 hour. There was a significant increase in threshold of hearing at 5 of 7 tested frequencies (4 kHz, $p<0.05$; 8 kHz, $p<0.001$; 11.3 kHz, $p<0.001$; 16 kHz, $p<0.001$; and 32 kHz, $p<0.0001$) at 1 month post damage and 6 of 7 tested frequencies (4 kHz, $p<0.01$; 8 kHz, $p<0.001$; 11.3 kHz, $p<0.0001$; 16 kHz, $p<0.0001$; 22.6 kHz, $p<0.001$ and 32 kHz, $p<0.0001$) at 3.5 month post damage. Degree of hearing loss remained relatively constant 1 month and 3.5 months after damage, implying that the threshold shift was present by 1 month and permanent. To determine if hearing loss was related to an animal's tinnitus status, we plotted the change in threshold of hearing as a function of frequency and compared changes between tinnitus positive and tinnitus negative animals. At 1 month post damage there were no statistically significant differences between the two groups (Figure 6B), although there does appear to be a trend toward a bigger change in the threshold of hearing at 4 kHz, 8 kHz and 16 kHz in tinnitus positive animals. Figure 6C shows that these differences diminish greatly at the 3.5 month time point, with change in threshold of hearing being virtually the same in tinnitus positive and tinnitus negative animals.

In order to better understand the relationship between hearing loss and tinnitus behavior we standardized the data by calculating Z scores for each measure and performed Pearson Correlations (Figure 6D, E, F). We narrowed down the relationships of interest based on our evaluation of all comparisons we performed. In examining the relationships between ABRs (8, 16 and 20k Hz) to gap detection performance (12, 16 and 20 kHz), we found strong positive

correlations between ABR threshold shifts measured at 8 kHz 1 month post damage and gap detection performance at 12 kHz ($r = 0.86$, $p = 0.01$) and 20 kHz ($r = 0.57$, $p = 0.07$). When there is a large change in ABR threshold there is greater impairment during gap detection performance. We did not find any strong relationships between ABR and GD at 3.5 months post damage. These results suggest that hearing loss at 8 kHz can predict gap detection performance 1 month following damage, but by 3.5 months that relationship is no longer apparent. Thus, the relationship between hearing loss and tinnitus is only apparent in the case of acute tinnitus.

Manganese enhanced MRI (spontaneous brain activity)

MEMRI changes in specific brain regions

To evaluate how sound damage impacts neuronal activity over time in auditory and non-auditory brain regions that have been implicated in tinnitus, we injected animals with manganese chloride and scanned their brain at baseline, 1 month post damage and 3.5 months post damage. This experimental design allowed us to follow individual animals over time and document hearing loss as a result of damage and timeline to tinnitus onset. Figure 7 shows our workflow for these experiments from scan to data analysis, including our use of the Paxinos and Watson atlas (6th edition) to identify ROIs. Data from three scanning sequences were used to calculate signal intensity. Figure 8 shows representative images as used for ROI placement for auditory structures.

Figure 9 shows changes in signal intensity 1 month (Figure 9A-C) and 3.5 months (Figure 9D-F) after exposure in three auditory brain regions (dorsal cochlear nucleus, inferior colliculus and auditory cortex). In this analysis, which does not separate animals based on

tinnitus status or follow specific animals over time, we observed no differences from baseline for DCN or auditory cortex at either 1 or 3.5 months. Sound damage did result in bilateral elevated signal intensity of the inferior colliculus ($p=.07$) 1 month following exposure. We did not see any changes in IC signal intensity at the 3.5 month time point (Figure D-F). For all regions we observed more variability at 3.5 months than at 1 month.

Our whole brain scanning allowed us to examine non-auditory regions thought to be involved in affective behaviors associated with tinnitus. Figure 10 shows representative images used for ROI placement of these non-auditory ROIs. Figure 11 shows changes in signal intensity for all animals 1 month (Figure 9A-C) and 3.5 months (Figure D-F) after damage in these regions (cerebellar parafloccular lobe, hippocampus including the dentate gyrus, and basolateral amygdala). With animals grouped together, sound damage did not result in any statistically significant changes at 1 month post exposure in any region. However, there does appear to be a trend toward increased activity in the contralateral BLA. Three and a half months following exposure, there was a bilateral decrease (IPSI, $p=.03$; CONTRA, $p=.06$) in signal intensity of the cerebellar parafloccular lobe. We did not see any significant changes in the hippocampal dentate gyrus or the basolateral amygdala. However, the trend we see in the contralateral BLA at 1 month post damage is still evident and appears to be stronger 3.5 months post damage. Within all the ROIs we measured, we observed greater variability in non-auditory brain regions than in auditory areas.

Table 2 shows calculated medians and p values based on Wilcoxon signed- rank test. All other MEMRI figure data are displayed as mean with standard error of the mean.

Again hoping to reveal any changes in signal intensity related to an animal's tinnitus grouping, we plotted the change in signal intensity from baseline after 1 month post damage and again at 3.5 months post damage as a function of brain region. In addition to identifying tinnitus status, this analysis evaluates individual animals over time. There were no statistically significant differences between tinnitus positive and tinnitus negative animals at any of the measured areas of interest, likely due to a small sample size. However, we did observe a few interesting trends in the data.

Figure 12 shows changes relative to baseline measured bilaterally in three auditory brain regions; auditory cortex (Aud Ctx), inferior colliculus (IC) and dorsal cochlear nucleus (DCN) according to tinnitus grouping at 1 month and 3.5 months post damage. At 1 month post damage (Figure 9B) there was an overall increase in signal intensity relative to baseline in the IC of both groups. Surprisingly, when animals are grouped according to tinnitus status (Figure 12A), we observed a greater increase in the ipsilateral IC of tinnitus negative animals relative to tinnitus positive animals ($p=0.26$). In the DCN, signal intensity changed differently in both groups, again with a surprising increase in the ipsilateral DCN of tinnitus negative animals relative to tinnitus positive animals ($p=0.25$). This trend continues at 3.5 months post damage (Figure 12 B), with an increase in SA in the ipsilateral and contralateral Aud Ctx of tinnitus negative animals relative to tinnitus positive animals ($p=0.20$). Due to the high variability in our data at 3.5 months post damage, we generated scatter plots indicating each animal's measured signal intensity in each ROI (Figure 12 C-E).

Figure 13 shows changes in signal intensity relative to baseline at 1 month and 3.5 months post damage in three non-auditory brain regions; hippocampus (Hippo), basolateral amygdala (BLA) and the parafloccular lobe of the cerebellum (PFL). One month after damage

(Figure 13 A) we observed a decrease in signal intensity in the ipsilateral PFL of tinnitus positive animals relative to tinnitus negative animals ($p=0.25$). Three and a half months post damage (Figure 13 B) changes from baseline were different between tinnitus positive and tinnitus negative animals, with there being a substantial bilateral increase in signal intensity in the hippocampus ($p=0.20$) and amygdala ($p=0.10$) of tinnitus negative animals relative to tinnitus positive animals. We included a scatter plot (Figure 13 C) of each animal's individual changes as a function of ROI because we saw more variability in our data at the 3.5 month time point. All changes were bilateral in non-auditory brain regions; therefore we grouped both sides together for each ROI.

Correlations between all measures

Given our small sample size, averages of changes in tinnitus positive or tinnitus negative animals are less likely to demonstrate changes in MEMRI label. However, because our experimental design includes repeated measures of multiple dependent variables in each animal, we can evaluate the relationships among our behavioral and physiological changes by performing correlation analyses.

Our remaining analysis examines correlations between hearing loss, tinnitus behavior and central nervous system spontaneous activity, measured by manganese uptake in selected ROIs, at 1 month and 3.5 months post damage. Sound damage was performed on the left ear so we focused on two different auditory pathways defined by synaptic connections (Direct; Ipsi DCN→Contra IC→Contra ACtx and Indirect; Contra DCN→Ipsi IC→Ipsi ACtx) when looking at relationships in auditory ROIs (Imig and Durham, 2005). These pathways are summarized in Chapter 1, Figure 3.

Figure 14A-B shows two strong correlations in the direct pathway relative to the site of peripheral insult at 1 month post damage. Change in ABR threshold at 8 kHz is positively correlated with signal intensity measured in the contralateral ACtx ($r = 0.67$, $p = 0.06$), and gap detection performance at 12 kHz is also positively correlated with signal intensity measured in the contralateral ACtx ($r = 0.74$, $p = 0.03$). These relationships suggest that greater hearing loss evident at 8 kHz and more pronounced tinnitus behavior at 12 kHz measured 1 month following sound damage may predict brain activity in the auditory cortex that receives direct input from the damaged ear.

Panels C-F in Figure 14 shows four strong correlations in the indirect pathway relative to site of sound damage at 1 month post damage. Increasing ABR thresholds at 8 kHz are correlated with signal intensity in the contralateral DCN ($r = 0.77$, $p = 0.003$). Likewise, worsening gap detection performance at 12 kHz is correlated with signal intensity measured in the contralateral DCN ($r = 0.81$, $p = 0.01$). These relationships suggest that hearing loss and tinnitus behavior may predict increasing spontaneous brain activity in the contralateral DCN (indirect pathway) at 1 month post damage, potentially compensating for lack of input from the ipsilateral (direct pathway) DCN to higher brain regions. At higher levels of the pathway, change in ABR thresholds at 16 kHz is negatively correlated with signal intensity measured in the ipsilateral inferior colliculus ($r = -0.49$, $p = 0.14$). Gap detection performance at 12 kHz is positively correlated with signal intensity in the ipsilateral ACtx ($r = 0.59$, $p = 0.09$).

At 3.5 months, post damage correlations were different than at 1 month. Within the direct pathway (Figure 15 A-C), signal intensity in the DCN (more proximal to the damage) increases as ABR thresholds at 16 kHz worsen in the ipsilateral DCN ($r = 0.76$, $p = 0.07$). In more rostral regions, change in ABR thresholds at 16 kHz ($r = -0.85$, $p = 0.03$) and gap

detection performance at 20 kHz ($r = -0.66$, $p = 0.15$) are negatively correlated with signal intensity in the contralateral auditory cortex.

In the indirect pathway (Figure 15 D-E), change in ABR thresholds at 8 kHz remains positively correlated with signal intensity measured in the contralateral DCN ($r = 0.82$, $p = <0.05$) as seen at 1 month. Correlations between gap detection performance and signal intensity in the ipsilateral auditory cortex are now altered both in direction (now a negative correlation) and with respect to GD frequency (now 20 kHz) ($r = -0.67$, $p = 0.14$).

Manganese signal intensity in the hippocampus shows an interesting relationship to gap detection at 1 month (Figure 16 A-B) that reverses direction at 3.5 months (Figure 16 C-E). At 1 month post damage there is a strong positive correlation between worsening gap detection performance at 12 kHz and increased signal intensity measured bilaterally in the hippocampus (Ispi: $r = 0.62$, $p = 0.08$ and contra: $r = 0.57$, $p = 0.11$). At 3.5 months after damage (Figure 15 C-E) worsening gap detection performance at 20 kHz and now predicts decreased signal intensity in the ipsilateral ($r = -0.73$, $p = 0.10$) and contralateral ($r = -0.77$, $p = 0.07$) hippocampus. A strong negative correlation between gap detection performance at 16 kHz now is also correlated with diminished signal intensity in the contralateral hippocampus ($r = -0.69$, $p = 0.13$).

Figure 17 shows the relationships we found between hearing loss, tinnitus behavior and spontaneous brain activity in other limbic areas including the basolateral amygdala and cerebellar parafloccular lobe at 1 month and 3.5 months post damage. As seen in the hippocampus, there was a strong correlation between worsening gap detection performance at 12 kHz and increased signal intensity in the ipsilateral basolateral amygdala ($r = 0.68$, $p =$

0.04) 1 month after damage. At 3.5 months following damage, the relationship reverses, with worsening gap detection performance at 16 kHz ($r = -0.74$, $p = 0.09$) and 20 kHz ($r = -0.79$, $p = 0.06$) now correlated with decreased signal intensity in the contralateral basolateral amygdala.

The cerebellar parafloccular lobe is the only non-auditory brain region in which brain activity was correlated with hearing loss as well as gap detection. Change in threshold at 8 kHz ABR (Figure 17D) was correlated with increased signal intensity in the contralateral parafloccular lobe at 3.5 months post damage ($r = 0.72$, $p = 0.10$), as was gap detection performance at 12 kHz with signal intensity in the ipsilateral parafloccular lobe ($r = 0.77$, $p = 0.10$).

A summary of these relationships can be seen in Table 3 (spontaneous activity and hearing loss) and Table 4 (spontaneous activity and tinnitus behavior).

Open Field Testing

One final test we ran on a subset of animals ($n=5$ at 1 month post damage, $n=3$ at 3.5 months post damage) is the open field test as a measure of anxiety behavior. We did not find any consistent differences in the open field testing scores themselves (time spent in the perimeter vs center in our testing box) between animals at different time points, likely due to our small sample size. We did, however find some interesting correlations between spontaneous activity in both auditory and non-auditory brain regions as reported by MEMRI and open field test behavior as shown in Figures 18 and 19.

At 1 month post damage there was a strong positive relationship between bilateral hippocampal signal intensity and open field test behavior (Figure 18A). Additionally there is a positive relationship between signal intensity in the ipsilateral basolateral amygdala and open

field test behavior (Figure 18B). These data suggest that as behavior evident of anxiety increases so does spontaneous activity in the hippocampus and basolateral amygdala. At 3.5 months post damage, the relationship between hippocampal and amygdalar activity changes direction and is negatively correlated with open field test behavior (Figure 18C-D). Interestingly, OFT relationships follow the same pattern as those for gap detection and MEMRI signal intensity in limbic areas; correlations are positive at 1 month and negative at 3.5 months. At this later time point there is a positive relationship between signal intensity in the cerebellar parafloccular lobe and open field test behavior (Figure E).

At 1 month post damage there was a strong negative correlation between ipsilateral (to the damaged ear) dorsal cochlear nucleus signal intensity and OFT behavior (Figure 19A). Conversely there is a strong positive correlation between the contralateral DCN and OFT behavior (Figure 19B). This suggests that as spontaneous activity decreases in the DCN receiving direct input from the damaged ear, so does anxiety behavior 1 month post damage. However, the reverse is true for the contralateral DCN. At 3.5 months post damage, these relationships weaken substantially (likely due to decreased sample size) but the direction remains the same (Figure 19C-D).

3.4 Discussion

MEMRI was a useful tool to measure changes in spontaneous activity non-invasively, allowing us to observe post damage alterations in multiple brain areas that occurred over time. Assigning sound damaged animals to tinnitus groups enabled us to determine if observed brain changes were related to hearing loss alone or tinnitus that occurs with hearing loss. Individual tinnitus assignment considered both baseline gap detection performance as well as

control performance relative to post damage performance adding strength to our sorting method.

Our longitudinal measures allowed us to demonstrate that gap detection scores from individual animals changed over time with 60% showing evidence of tinnitus behavior 1 month after damage and 45% 3.5 months post damage. While animals differed in tinnitus status, hearing loss by and large occurred in all animals. We demonstrated hearing loss at all tested frequencies > 4 kHz, with hearing loss measured at 1 month still evident 3.5 months post damage. More severe hearing loss was present at 4 and 8 kHz in the tinnitus positive group 1 month following damage, but these differences were no longer evident 3.5 months after damage. Despite little difference in hearing loss between tinnitus groups, severe hearing loss at 8 kHz was correlated with impaired gap detection performance at 12 and 20 kHz. Hearing loss at 16 kHz was also correlated with impaired gap detection at 20 kHz. Hearing loss was more significant which will be discussed below.

Our evaluation of spontaneous activity (SA) with MEMRI without regard to tinnitus status did reveal changes in SA that might be anticipated following sound damage. We observed bilateral increases in spontaneous brain activity in the inferior colliculus at 1 month and decreased spontaneous activity in the parafloccular lobe, with higher variability at 3.5 months post damage. Unexpectedly, when animals were sorted according gap detection performance, increased SA was only seen in tinnitus negative animals with the exception of the PFL. Here we observed decreased SA in both tinnitus negative and positive animals and these changes only become evident at 3.5 months post damage. Our ability to assign tinnitus status and hearing loss to individual animals enabled this analysis. When we looked at the relationship between hearing loss, tinnitus behavior and brain activity we observed that ABR

and GD performance at specific frequencies can predict spontaneous brain activity in both auditory and non-auditory brain areas. The direction of the correlations changed over time, suggesting unique patterns of activity in both auditory and non-auditory brain regions for each time point.

Gap detection and tinnitus behavior

As reported elsewhere (Engineer et al 2011, Kraus et al 2010, Pace & Zhang 2013) we observed sound-induced tinnitus behavior in roughly 50% of animals, with identification based on their gap detection performance. Our study design enabled us to determine if an animal's tinnitus status remains unchanged over time. We found that tinnitus status changed in 63% of animals by 3.5 months post damage, signifying the importance of relevant timeline establishment during future studies. These findings also suggest that in some cases there may be a limited time window after damage occurs that animals can be rescued and/or prevented from developing tinnitus behavior.

Since the development of the reflex-based gap detection test (Turner et al 2006), its reliability has become a controversial subject. For experiments with limited timelines, gap detection testing is a valuable option because it does not require time for training as interrogative methods do. However, some argue that the strengths inherent in interrogative methods (reliance on auditory perception, similarity to detection of tinnitus in humans, ability to reveal functions from many brain areas) are absent from reflexive methods such as gap detection (Brozoski & Bauer 2016, Lobarinas et al 2013b). Additionally, Lobarinas and colleagues (2013) have suggested that the test may result in false positives since unilateral hearing loss can cause disrupted responses during startle reflex testing, regardless of tinnitus

status. Lastly, gap detection results in a wealth of data and there has been a lack of consistency and agreement amongst researchers about how best to analyze and sort the data, perhaps contributing to variable published results.

We modified the startle gap detection test and its analysis to address concerns over its ability to identify animals with tinnitus. In our experiments, each animal serves as its own control since we measure gap detection performance at baseline and then again at each post damage time point. For our analysis we normalized each animal's testing performance following sound damage (1 and 3.5 months) relative to their performance at baseline. This allowed us to determine if differences result from within-subject or between-subject variability. Additionally, testing is done over 4 days and performance is calculated day by day prior to calculating a final gap detection score at the end of the testing period. By doing this we hope to produce data sets that represent more consistent performance rather than testing at one given day and time. Furthermore, we used the Grubbs outlier test to identify a maximum of one outlier per data set in an effort to decrease the likelihood of an extreme responder skewing results. Lastly, we collected control gap detection data at baseline and then again approximately 1 month following a wait period to understand how gap detection performance changes due to nothing more than time between testing periods. We used these data to generate heat maps based on each animal's performance relative to controls and then sorted animals into tinnitus groups. This method of sorting goes above and beyond other methods whereby criterion for evidence of tinnitus behavior is defined by a gap score of >1 in damaged animals and does not account for changes that may be present in control animals. Given this method of analysis we feel confident our startle testing provides a reliable measure of tinnitus status.

Auditory brainstem response and hearing loss

The sound damage paradigm used to induce hearing loss in this study is milder in intensity and duration than other paradigms we have used in past experiments. Neal et al. (2015) documented less hair cell loss with the 114 dB sound damage paradigm vs. a more intense 118 dB, 4 hour damage (Neal et al 2015). Based on previous work we did not expect to see significant shifts in hearing threshold at every frequency. Kennon-McGill observed a significant threshold shift only at 16 kHz when using this same mild sound damaging stimulus (Kennon-McGill 2014). However it is important to note that hearing loss reported here shows threshold shifts at 1 month and 3.5 months post damage relative to baseline. These differences highlight the importance of determining baseline hearing thresholds for each individual animal prior to damage thereby providing a more accurate picture of how hearing declines over time.

With the exception of 2 kHz and 22.6 kHz, we observed significant hearing loss at five of the seven tested frequencies at 1 month post damage. These threshold shifts remained relatively stable over time, with the exception of 22.6 kHz which becomes significant only at 3.5 months; the stable thresholds suggest a permanent shift. The most robust shift from baseline to 3.5 months was at 16 kHz with a 20 dB shift. It's important to note that sound exposure was done unilaterally and hearing was still intact in the unexposed ear. Additionally, ABR absolute threshold data shows that all animals responded to stimuli >50 dB even in their damaged ear. Given these absolute thresholds, animals should be able to respond to background stimuli presented at 60 dB during gap detection testing.

When changes in ABR thresholds were compared between tinnitus negative and tinnitus positive animals, while not significant, we saw greater shifts in tinnitus positive animals at 4 and 8 kHz. We observed a strong relationship between more severe hearing loss at 8 kHz and gap detection performance at 12 and 20 kHz. A similar relationship exists between hearing loss at 16 kHz and impaired gap detection performance at 20 kHz. Hearing loss and gap detection performance are only correlated at 1 month post damage, suggesting that degree of hearing loss may predict gap detection performance but only in the early cases of tinnitus onset.

While this correlation analysis revealed numerous strong relationships between measures employed in this experiment, it is important to note that the correlation between two variables does not imply that the change in one variable is *caused* by the other variable. Correlation data gives us the ability to *predict* how variables interact, providing a framework from which to design experiments to investigate potential causality.

Manganese enhanced MRI and spontaneous brain activity

Previous studies have reported increased spontaneous brain activity (SA) in various auditory brain areas following sound damage and have proposed increased SA as a neural correlate of tinnitus. Increases have been reported in the DCN (Brozoski et al 2002, Chang et al 2002, Kaltenbach et al 2000, Kaltenbach et al 2005), IC (Bauer et al 2008, Dong et al 2010, Mulders & Robertson 2009) and auditory cortex (Huetz et al 2014, Norena & Eggermont 2003, Seki & Eggermont 2003). However, other groups have reported no change using electrophysiological recording methods (Ma & Young 2006) or even a decrease in activity using the 14C-2deoxyglucose (2DG) metabolic assay (Imig & Durham 2005). These seemingly

contradictory findings may be due to differing species, differences between sound damage paradigms as well as varying limitations in each measurement (e.g. anesthesia use, single cell measurements within a brain region, activity of an entire brain region, etc.). Electrophysiology is a measure of neuron electrical activity, mainly action potentials, and recordings can be done in a single cell or a cluster of cells. The ^{14}C -2deoxyglucose assay measures glucose uptake in neurons and greater 2DG accumulation implies greater activity. While both methods are measures of brain activity, they are mechanistically distinct as electrophysiological recordings are reflective of discharging neurons (dependent on action potentials) and 2DG density is more likely to reflect active synapses (does not depend on action potentials) (Nudo & Masterton 1986). Additionally it has been shown that anesthesia usage can also affect measures of spontaneous activity (Kennon-McGill 2014).

A more recent tool used to measure spontaneous brain activity related to tinnitus or in general is manganese-enhanced MRI (MEMRI). Manganese, a calcium analog, enters active cells via voltage gated calcium channels. The cellular basis of the MEMRI signal is thought to involve neuron-glia interactions in the presence of neuronal injury. Bade and colleagues (2013) reported that astrocytic reactions stimulate neuronal Mn^{2+} ion uptake (while Mn^{2+} content in glial cells was not related to glial activation) thereby resulting in MEMRI signal enhancement (Bade et al 2013). Manganese chloride serves as a long-lasting contrast agent when injected prior to the MRI scan. Regions with high activity accumulate more manganese than less active regions and more manganese is equivalent to greater signal intensity in MR images. Importantly, neither anesthesia nor the sound of the scanner should impact the results, as manganese is injected prior to scan and uptake occurs under ambient noise conditions in freely moving animals. This method of measuring spontaneous activity gave us

the advantage of being able to look at several ROIs in the same animal and at multiple time points.

Changes to MEMRI brain activity in salicylate and noise induced tinnitus models have been reported in several recent studies, indicating differences we did not observe in our experiments (Table 1). For example, elevated activity has been reported in several brain regions, including the cochlear nucleus (Brozoski et al 2013, Groschel et al 2016, Holt et al 2010), inferior colliculus (Brozoski et al 2007, Groschel et al 2016, Holt et al 2010), auditory cortex (Groschel et al 2016), parafloccular lobe (Brozoski et al 2007, Brozoski et al 2013) and amygdala (Groschel et al 2016) following both salicylate and noise to induce damage and tinnitus. Here we report that one month following damage only one out six measured regions, the inferior colliculus, reflected increased spontaneous activity. These changes were acute, as by 3.5 months post damage activity was close to baseline levels. Brozoski et al. (2007) reported general decreases in activity of forebrain areas (medial geniculate nucleus, auditory cortex and amygdala) following sound damage, while the only decreased activity observed in our study was in the PFL three and half months after damage occurred. As shown in Table 1, the only tinnitus study that measured SA in regions outside the auditory system with MEMRI at a later time point post damage was done by Brozoski et al. (2007). This study measured manganese uptake ex vivo, as animals were sacrificed and decapitated prior to imaging, 10 months after damage. Increased SA was reported in the PFL and IC while decreased activity was reported in the auditory cortex and amygdala relative to controls 10 months following damage. While ex vivo measurements should not influence manganese uptake since uptake occurred over a 24 hour period in awake animals, it is a methodological difference between this study and others listed in Table 1 worth noting. In contrast to our study, comparisons were

made between control animals (no sound damage) and sound damaged animals while we compared baseline activity relative to post damage activity in all ROIs

The utilization of salicylate has been shown to produce tinnitus reliably in treated animals. In contrast, tinnitus induction by way of exposure to damaging sound has produced variable degrees of tinnitus in animals exposed to the same stimulus, thereby indicating the importance of using some behavioral method to identify attributes of tinnitus behavior that may not be present in all animals. None of the aforementioned studies sorted damaged animals into tinnitus subgroups, while our results indicate that sorting animals into groups based on gap detection performance can have a dramatic effect on the outcome. Interesting trends were observed when comparisons were made between tinnitus subgroups. Tinnitus negative animals displayed increased SA in the DCN and IC, lower regions of the auditory pathway, at 1 month post damage. At the later time point, three and a half months after damage, we observed increased SA in the auditory cortex, a high auditory region. The altered spontaneous activity observed in the IC at one month (increased relative to baseline at) in combination with findings reflected when animals were sorted by tinnitus group at one month (tinnitus negative animals had increased SA relative to tinnitus positive) suggests that increased SA in the IC may be a result of sound induced hearing loss that occurs in the absence of tinnitus. Observed decreases in SA of the PFL at three and a half months (decreased relative to baseline) when all sound damaged animals were grouped together were reflected in both tinnitus negative and tinnitus positive animals (decreased activity) when comparisons were made between tinnitus groups suggesting that sound damage induced similar changes in all animals that were not unique to a specific tinnitus group. While we didn't see any changes in measured activity of limbic brain regions when all animals were grouped together, we

observed decreased SA within the hippocampus and basolateral amygdala of tinnitus positive animals at three and a half months post damage indicating that tinnitus related neuroplastic changes in limbic brain regions take some time to occur.

Comparing MEMRI results across groups relative to our findings further supports the somewhat recent realization of tinnitus generation and manifestation complexity in that several brain areas are involved, increases and decreases in activity are present, method of tinnitus grouping (or lack thereof) and variables such as time from injury (so-called tinnitus induction) to measurement can significantly influence the outcome.

Relationships between hearing loss and changes in spontaneous brain activity

In this experimental design we were able to measure how sound damage impacts hearing loss, tinnitus behavior, changes in brain activity and anxiety behavior in the same animals over time. Due to the strength of this design we were able to assess how all these measurements are related and how they change over time using correlation analyses.

Hearing loss measured at 8 kHz (1 month post damage) and 16 kHz (3.5 months post damage) may serve as a predictor of spontaneous brain activity in various auditory brain regions that receive both direct and indirect input from the damaged ear. The majority of relationships observed between hearing loss and SA were positive, meaning that more severe hearing loss indicated increased SA. The exceptions to this were in the inferior colliculus (indirect pathway) at 1 month post damage and the auditory cortex (direct pathway) at 3.5 months in which more hearing loss indicated decreased spontaneous brain activity. More severe hearing loss at 8 kHz was associated with increased SA in the DCN receiving indirect input from the damaged ear and this relationship persisted over time. Perhaps the

contralateral DCN is compensating for loss of input from the site of damage (Roberts et al 2010). There is hemispheric cross-talk via commissural fibers between both sides of the DCN (shown in Chap. 1, Figure 3) that enable the DCN to receive binaural auditory information (Brown et al 2013). It's been shown that commissurals often have an inhibitory effect on their targets. Disrupted input through commissural fibers from ipsilateral (damaged side) to contralateral DCN could play a role in the relationship observed here and be compensatory in nature. It is not until 3.5 months after damage that we see a relationship between hearing loss and the DCN directly impacted by sound damage ($>$ hearing loss at 16 kHz \rightarrow $>$ SA ipsi DCN). This observation highlights findings found by (Guitton & Dudai 2007) suggesting that there is some time window during which neuroplastic changes take place in the DCN.

The cerebellar paraflocculus was the only non-auditory brain region for which MEMRI label was strongly correlated with hearing loss (vs. tinnitus). While the PFL is generally thought to be involved in motor control and tasks such as the vestibulo-ocular reflex, the PFL has been shown to respond to sound (Lockwood et al 1999). Additionally, evidence has shown that there is direct input from the cochlear nucleus to the PFL (Morest et al 1997) and that the PFL receives descending information from the IC and auditory cortex (Bauer et al 2013a, Chen et al 2017). More severe hearing loss at 8 kHz was associated with increased SA in the contralateral PFL at 3.5 months. Interestingly, this relationship mirrors the relationship we observed in the contralateral DCN 3.5 months following damage. The degree to which the DCN activity is disrupted by sound damage could similarly impact the PFL due to the direct connection from the DCN to the PFL and because the PFL has been shown to respond to sound stimuli.

Relationships between tinnitus behavior and spontaneous brain activity

Given our unilateral damage paradigm, we can assign auditory structures as belonging to either the direct or indirect pathway, relative to location from damaged ear. As shown in Chapter 1, Figure 3, the direct pathway includes the ipsilateral dorsal cochlear nucleus, contralateral inferior colliculus and contralateral auditory cortex. One month following damage, more robust gap detection scores at 12 kHz are associated with increased spontaneous activity in both the dorsal cochlear nucleus (indirect) and auditory cortex (direct and indirect). Recall that gap detection testing at 12 kHz yielded improved performance for almost all animals, with only a few exceptions. This means that more robust gap detection scores do not always imply tinnitus behavior; instead they indicate performance above and below the mean (of all damaged animals), regardless of tinnitus status. 3.5 months following damage, more robust gap detection performance at 20 kHz was associated with bilaterally decreased SA in the auditory cortex. At this later time point only 1 animal was identified as having tinnitus at 20 kHz, thus this relationship also does not appear to be dependent on tinnitus status.

The relationships between gap detection performance and spontaneous activity in limbic brain regions yielded interesting findings that change over time. More pronounced 12 kHz gap detection scores at 1 month were associated with increased SA in ipsilateral basolateral amygdala and bilateral hippocampus 1 month post damage. At 3.5 months post damage the direction of these relationships changes ($>$ gap detection scores \rightarrow lower SA) and occurs at both 16 kHz and 20 kHz gap detection testing backgrounds. The direction of the relationship between gap detection performance and SA went from positive to negative in the span of two and a half months. Animals with more impaired gap detection performance had increased SA in the short term that changed to decreased SA by 3.5 months post damage.

These results are similar to what we see when we separated animals into tinnitus groups and compared mean changes to SA at 3.5 months post damage. This suggests that limbic brain regions are more active in tinnitus negative animals (lower gap detection scores) at 3.5 months post damage.

Relationships between anxiety behavior and spontaneous brain activity

More anxiety was associated with increased SA in bilateral hippocampus and ipsilateral basolateral amygdala, with a stronger relationship evident in the hippocampus. Anxiety provoking stimuli presented to patients in a human imaging study also reported increased amygdalar and hippocampal activity (Martin et al 2009). Again, the direction of this relationship changes from positive at 1 month post damage to negative at 3.5 months post damage. This suggests that our sound damage paradigm results in increased brain activity in the hippocampus and amygdala that coincides with evidence of anxiety behavior in the short term but over time this relationship reverses, whereby more activity is correlated to less anxiety. A recent MEMRI study on the effects of blast-induced tinnitus by Ouyang et al. (2017) reported increased MEMRI signal in limbic brain regions in association with increased anxiety five weeks after blast exposure(Ouyang et al 2017) which supports our findings 1 month post sound exposure. More studies are needed to better understand why the direction of these relationships may change over time.

Our experimental design allowed us to determine if subsequent measured changes were indicative of hearing loss alone or hearing loss and tinnitus at both time points. Additionally, we found that tinnitus behavior can change over time. Hearing loss trended toward more severe at 4 and 8 kHz in tinnitus positive animals at 1 month post damage. When

MEMRI signal was averaged across all animals we found differing results than we did when animals were sorted based on evidence of tinnitus. Using averages of all animals we saw increased activity only in the auditory IC at 1 month post damage. Three and half months after damage we saw decreased activity only in the non-auditory PFL when all animals were averaged together. Interestingly when animals were sorted by tinnitus group, tinnitus negative animals are the ones that show increased SA in the IC at 1 month suggesting that tinnitus status may contribute to these differences. However decreased SA in the PFL at 3.5 months appears to result from hearing loss alone as both tinnitus positive and negative animals showed decreased SA in the PFL.

From this longitudinal experiment we can conclude that exposure to mild damaging sound can induce tinnitus in approximately half of animals regardless if behavior was measured at 1 month or 3.5 months following damage. We also showed that tinnitus behavior can change over time, suggesting that tinnitus behavior should be monitored over time when seeking to compare other measures at different time points. In contrast to what we observed with tinnitus behavior, sound damage induced hearing loss holds steady over time with hearing loss being more severe in tinnitus positive animals 1 month post damage. Therefore, hearing loss persists while tinnitus behavior is in flux.

Outcomes of spontaneous activity measurements, such as MEMRI, can change dramatically depending on the specificity of groups being compared. Differential measured changes are ROI and time point dependent. Consideration of how two measured variables are related (both strength and direction of relationship) is important when interpreting results but is often overlooked in tinnitus research studies. Hearing loss and gap detection performance can aid in predicting changes in spontaneous brain activity in both auditory and non-auditory brain

regions. While the complexity and breadth of CNS regions implicated in tinnitus are major hurdles in the realm of auditory research, understanding how different variables relate to one another in different animal models can serve to pinpoint areas and changes that warrant a more in depth investigation.

Table 1: Comparison of MEMRI studies

MEMRI study comparison							
Stimulus	Manganese dose and time before imaging	MRI field strength/ Imaging Assessment	Post-stimulus MRI	Species	Brain Regions with changes in Activity	Tinnitus Assessment	Reference
16 kHz, 115 dB SPL, 1 h	20 mg/kg (IP), 24-48 h	14.1 T / Ex vivo	10 months	Long-Evans Rat (Male)	A1, Amyg, IC, LL, MGB, PFL, PVCN	Operant-conditioned suppression	Brozoski et al., 2007
10 kHz, 1/3 octave, 118 dB SPL, 4 h	66 mg/kg (IP), 8 h	4.7T / In vivo	48 h	Sprague-Dawley Rat (Male)	DCIC	Gap Detection	Holt et al., 2010
Salicylate 300 mg/kg IP daily for 3 days	66 mg/kg (IP), 8 h	4.7 T / In vivo	8 h after 3rd injection	Sprague-Dawley Rat (Male)	CNIC, DCIC, DCN, ECIC	Gap Detection	Holt et al., 2010
16 kHz, 120 dB SPL, 1 h	88 mg/kg (SC), 20-28 h	14.1 T / Ex vivo	3 months	Long-Evans Rat	DCN, PVCN	Operant-conditioned suppression	Brozoski et al., 2013
Salicylate 250 mg/kg IP after kanamycin-induced hair cell damage	0.4 mM/kg (IP), 24 h	7T / In vivo	24-72 h	NMRI Mice	AC, Amyg, DCN, IC, MGB, SOC, VCN	None	Groschel et al., 2016
Single free-field blast	67 mg/kg (IP), 8 h	7T / In vivo	5 weeks	Sprague-Dawley Rat (Male)	AC, Amyg, DCIC, DCN, ECIC, MGB, NAc, VCN	Gap detection	Ouyang et al., 2017
16 kHz, 114 dB, 1 h	66 mg/kg (IP), 24 h	9.4T / In vivo	1 month and 3.5 months	Long-Evans Rat (Male)	IC (1 month) PFL (3.5 months)	Gap detection	Freemyer et al., 2018 (in progress)

Table 1. Noise, salicylate or blast induced tinnitus and the impact on brain activity as measured by MEMRI in different rat and mouse species. Abbreviations: AC, auditory cortex; A1, primary auditory cortex; Amyg, amygdala; CNIC, central nucleus of inferior colliculus; DCIC, dorsal cortex of inferior colliculus; DCN, dorsal cochlear nucleus; ECIC, external cortex of inferior colliculus; IC, inferior colliculus; IP, intraperitoneal; LL, lateral lemniscus; MGB, medial geniculate body; NMRI (mice); NAc, nucleus accumbens; PFL, parafloccular lobe of cerebellum; PVCN, posteroventral cochlear nucleus; SOC, superior-olivary complex; VCN, ventral cochlear nucleus.

Figure 1. Experimental Timeline

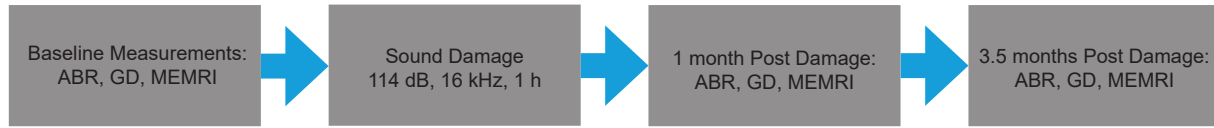


Figure 1. Experimental timeline used to determine effect of sound damage on spontaneous brain activity at acute (1 month) and chronic (3.5 months) time points post damage. ABR: auditory brainstem response, GD: gap detection, MEMRI: manganese-enhanced MRI, OFT (open field test)

Figure 2. Gap Detection Controls

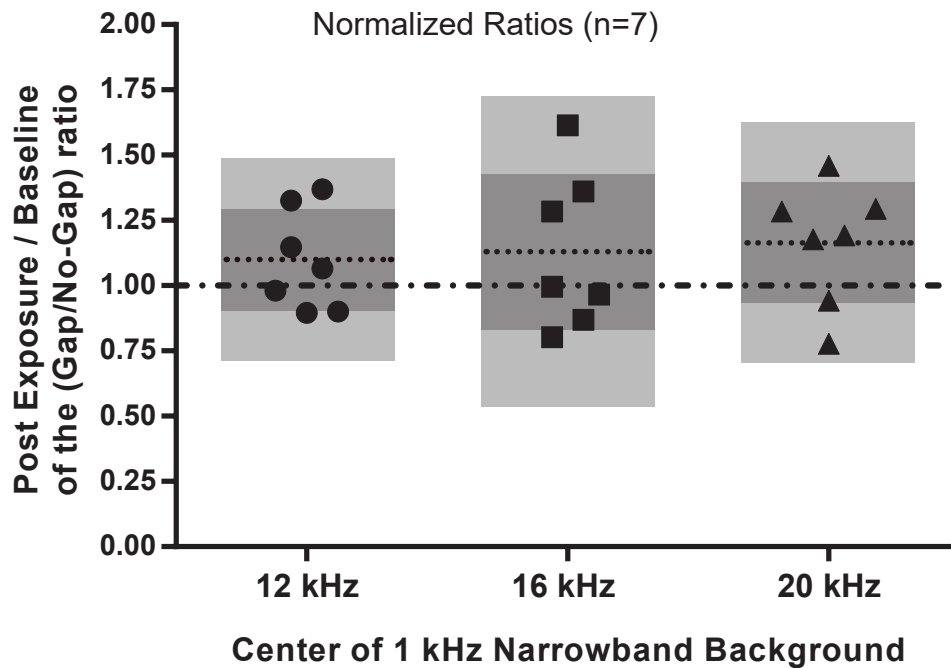


Figure 2. Gap detection data obtained from control (no sound damage) animals. Normalized ratios [post waiting period/baseline of the (gap/no gap) ratio] from each individual animal are plotted as function of background noise (12, 16, 20 kHz). The dotted line is the mean of all animals at a given frequency. The shaded gray bars represent standard deviation of the mean (light gray: 2 standard deviations, dark gray: 1 standard deviation).

Figure 3. Gap Detection 1 month PD

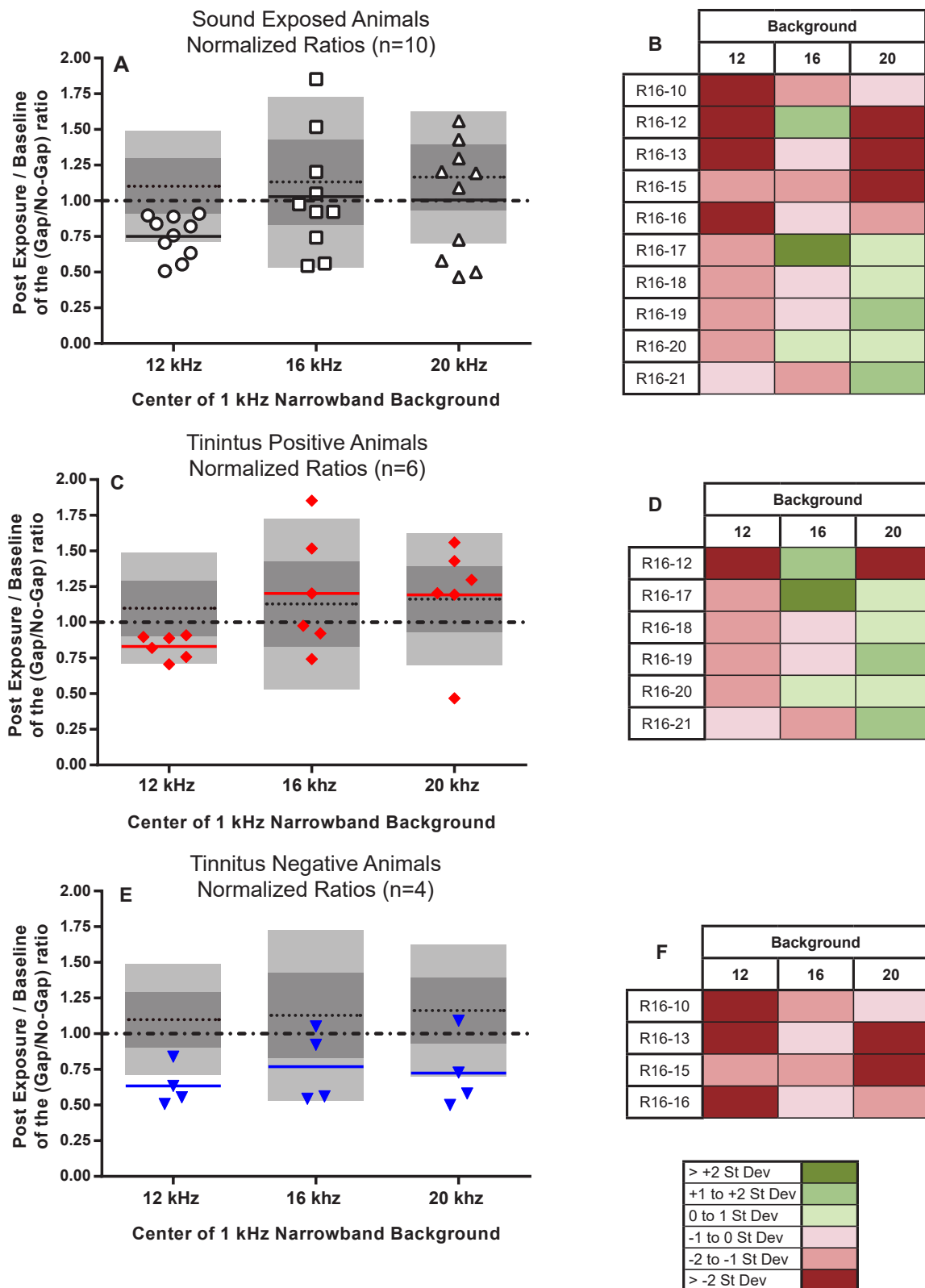


Figure 3. Gap detection data obtained 1 month after animals were sound damaged. A,C,E show the performance of sound damaged animals overlaid on the range of control standard deviations (shaded gray bars and dotted lines as in Figure 2). The solid line in each graph is the mean of data obtained from sound damaged animals. Normalized ratios are plotted as a function of background frequency of ALL sound exposed animals (A). Heat maps (B, D, F) were generated to show each individual animal's performance relative to controls. Dark green to light green was used to indicate impairment in performance relative to controls (GD score $>+2$, $2-1$ or less than 1 SD above controls) and dark red to light red was used to indicate improvement in performance relative to controls (GD score >-2 , $1-2$, or less than 1 SD below controls). This heat map was used to sort animals into tinnitus positive (impaired performance at one or more frequencies) and tinnitus negative (improved performance at all 3 frequencies) groups. Normalized gap detection ratios are plotted as a function of background frequency along with their corresponding heat maps for tinnitus positive (C, D) and tinnitus negative animals (E, F).

Figure 4. Gap detection 3.5 months post exposure

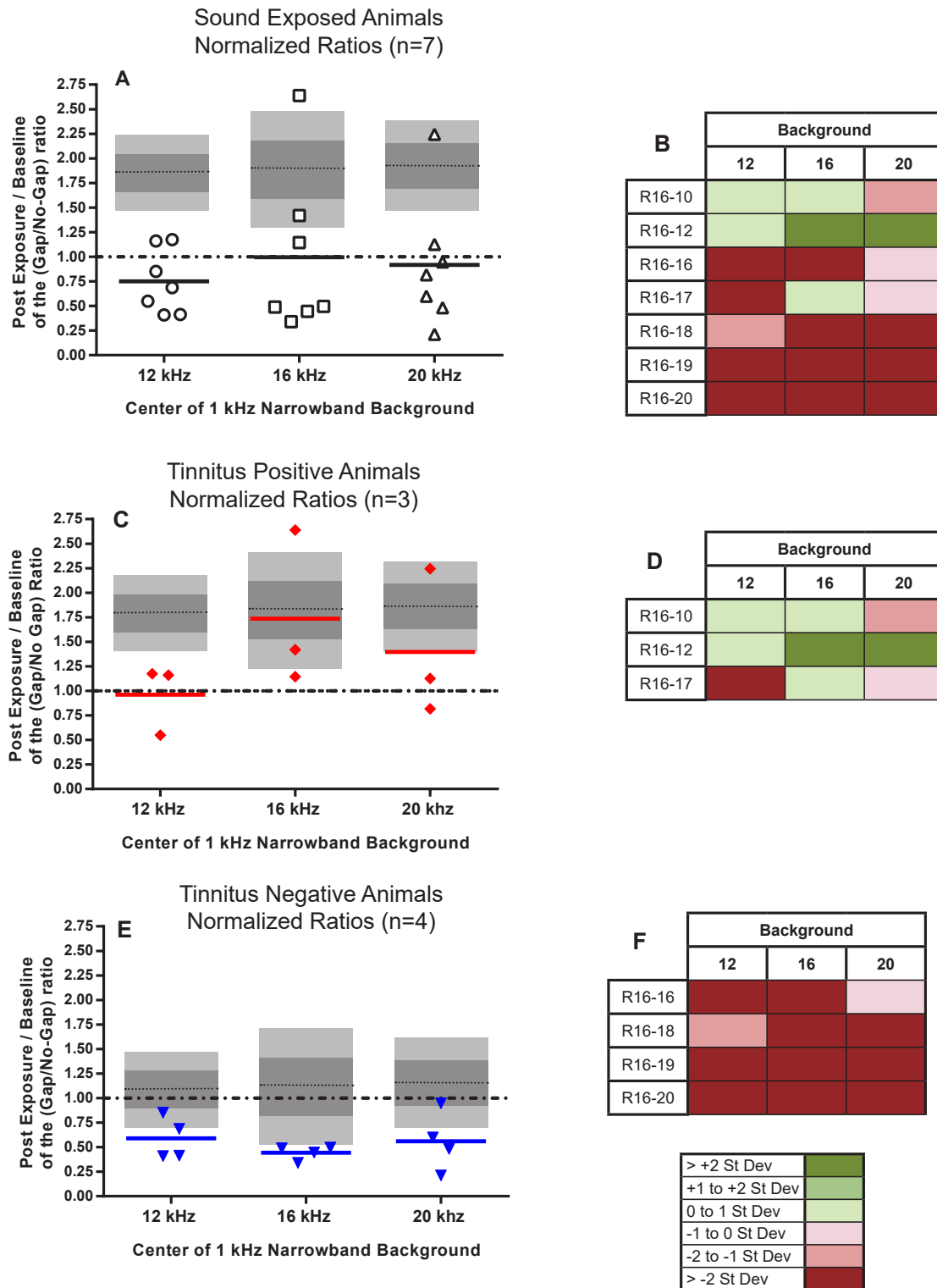
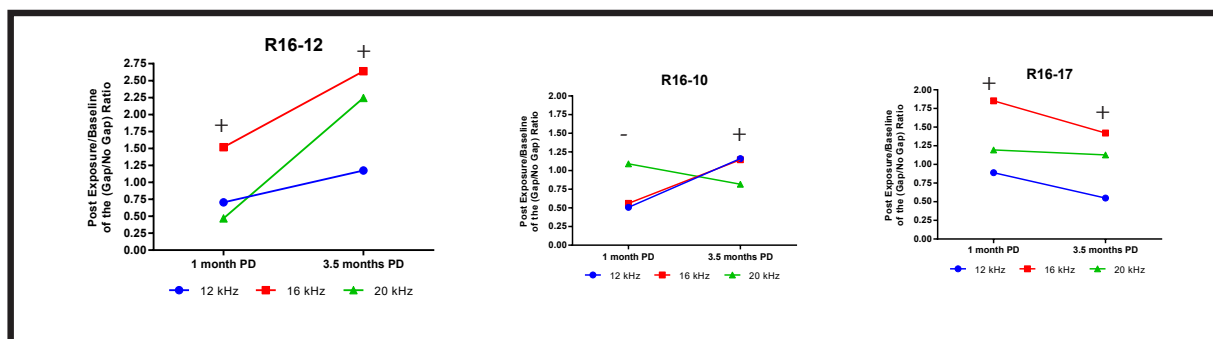


Figure 4. Gap detection data obtained 3.5 months after animals were sound damaged. Conventions for plotting GD scores (A, C, E) generating heat maps (B, D, F) and assigning animals as tinnitus positive or negative are the same as in Figure 3. Control data used for comparison are from the same animals, evaluated at 1 month post baseline. The change in sample size is due to animal deaths prior to the 3.5 months post damage time point.

Figure 5. Identifying Tinnitus Group 3.5 Months Post Damage Based on Change in Gap Detection Performance of Individual Animals

Tinnitus Positive at 3.5 Months



Tinnitus Negative at 3.5 months

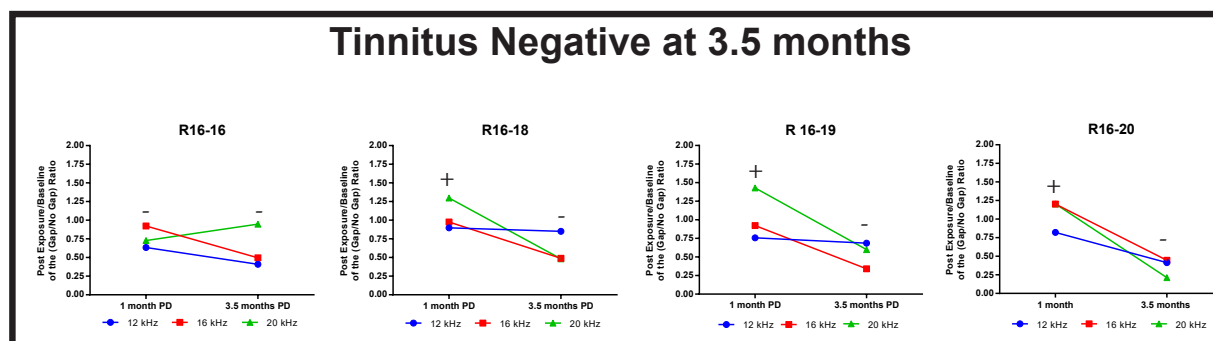


Figure 5. Identification of tinnitus group 3.5 months post damage based on change in gap detection performance over time in each individual animal. The top three graphs show animals identified as tinnitus positive and the bottom four graphs show animals identified as tinnitus negative at 3.5 months post damage. Tinnitus status of individual animals can be tracked over time (+ indicates tinnitus positive and – indicates tinnitus negative).

Figure 6. Auditory Brainstem Response, Hearing Loss and the Relationship with Tinnitus Behavior

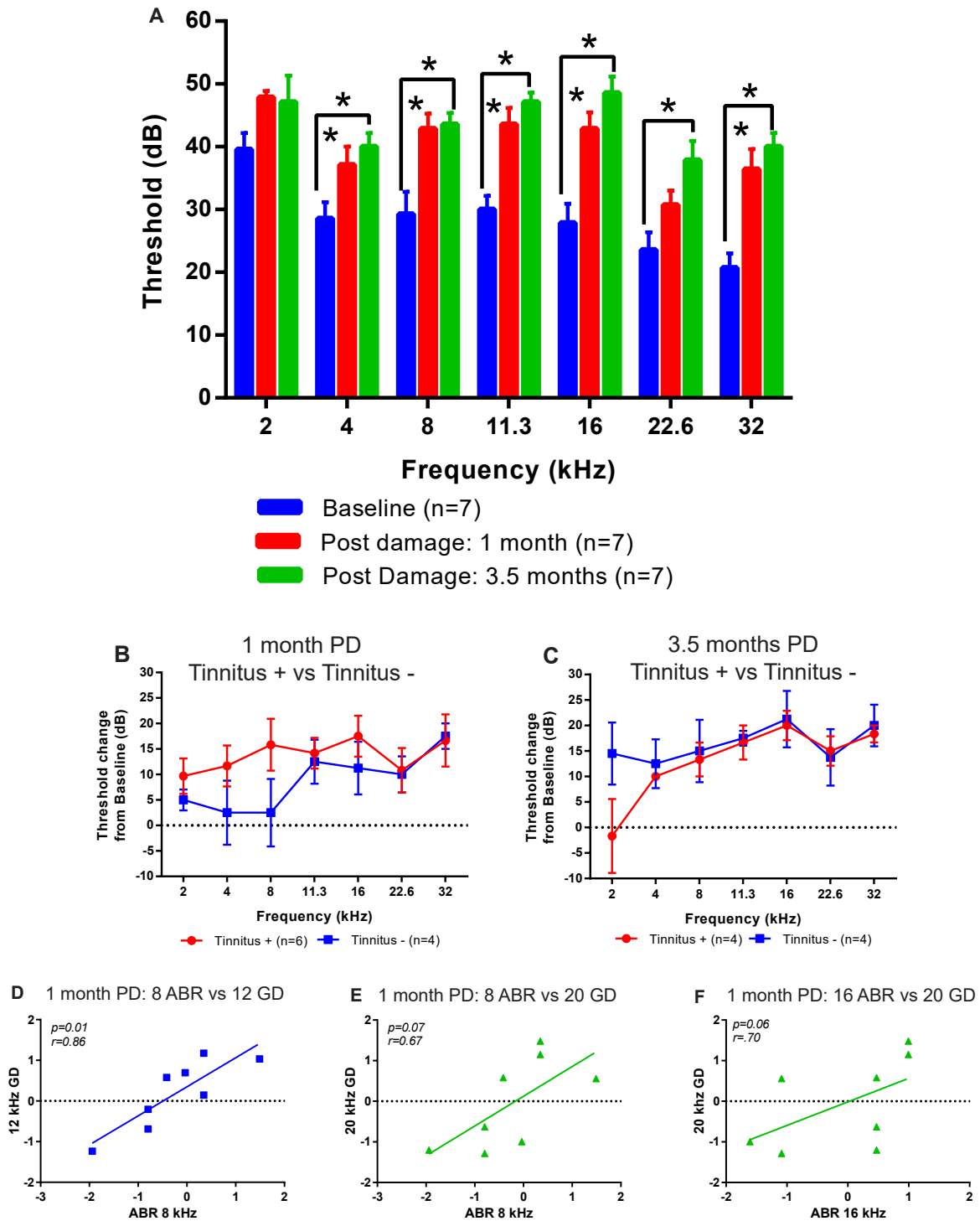


Figure 6. Auditory brainstem response, hearing loss and the relationship with tinnitus behavior. In A the mean hearing threshold (\pm standard error of the mean) for all animals at baseline, 1 month post damage and 3.5 months post damage is plotted as a function of frequency. Asterisks indicate significant differences (two way-repeated measures ANOVA, Tukey multiple comparisons). Degree of hearing loss remained constant over time. Change in threshold of hearing for tinnitus positive and tinnitus negative animals at 1 month (B) and 3.5 months (C) post damage is plotted as a function of frequency. No significant differences were observed. D, E & F are correlations between ABR Z scores and GD Z scores at 1 month post damage (Pearson Correlation, $p < .05$).

Figure 7. Workflow from MRI scan to results

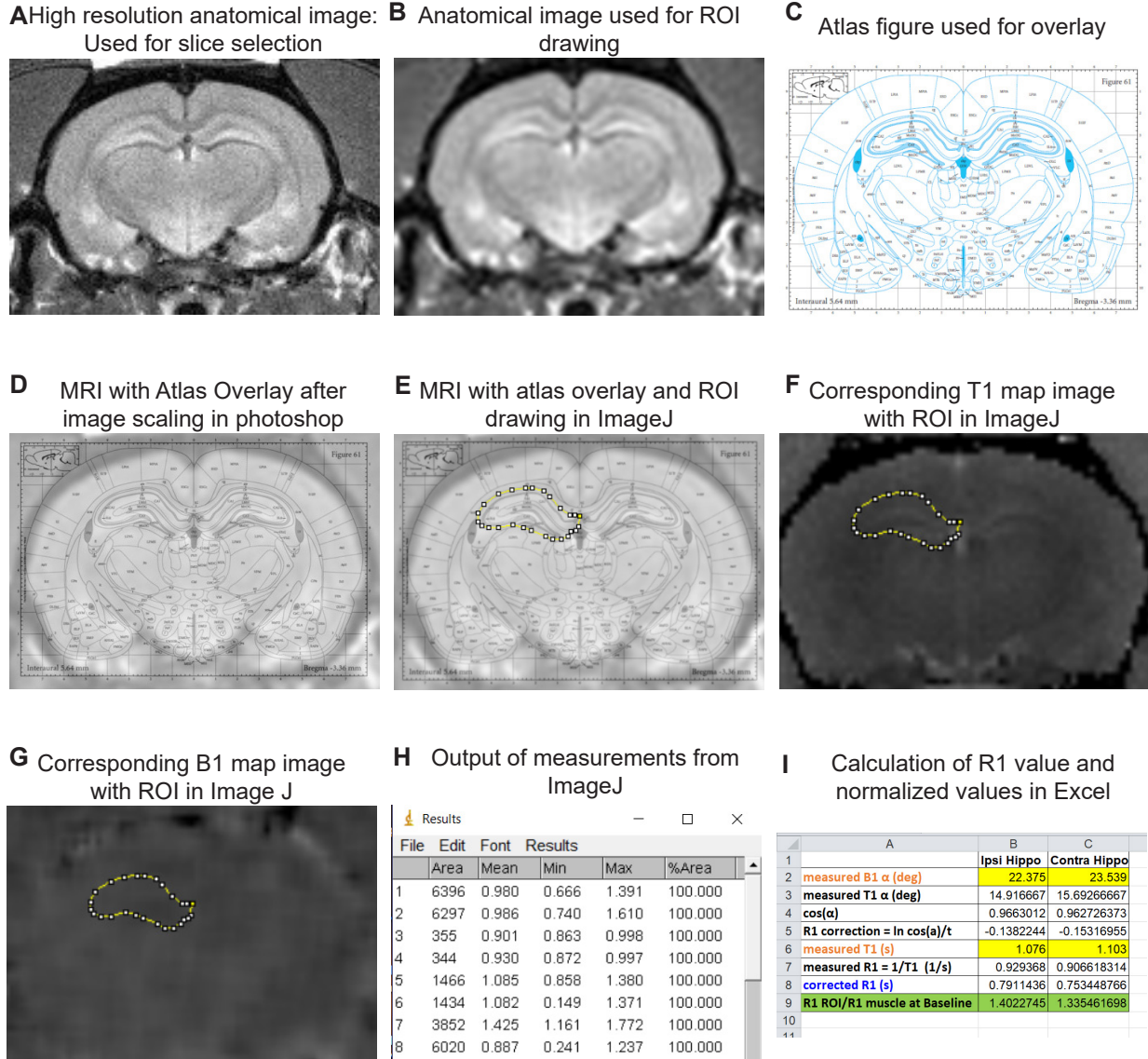


Figure 7. Workflow from MRI scan to final results. High resolution images (A) were used to select the section that best represents a given region of interest (ROI; e.g. hippocampus). Once a slice was chosen, we used the corresponding lower resolution MRI to outline the ROI (B), using the digital Paxinos and Watson rat brain atlas (C). The atlas image is next overlaid on the MRI section (D) and the ROI is outlined (E). Using these ROIs we used ImageJ to measure signal intensity on T1 (F) and B1 (G) images generated during the scans. Signal intensity was measured using ImageJ (H) and corrections and data normalization were done in Excel (I).

Figure 8. MEMRI Representative Images: Auditory ROIs

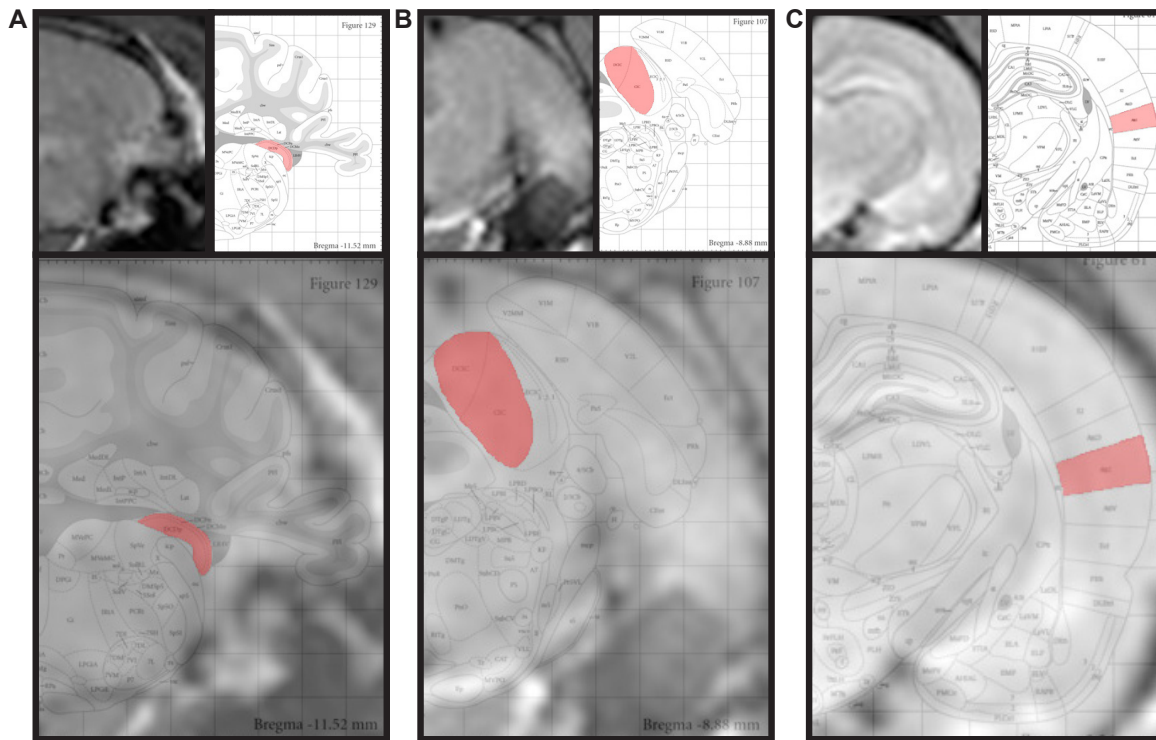


Figure 8. Representative MEMRI images of auditory regions of interest. The dorsal cochlear nucleus (A), inferior colliculus (B) and auditory cortex (C) were outlined to measure spontaneous brain activity. In each panel the top left image is the anatomical MRI, top right image is the atlas and the bottom is the MRI/atlas overlay.

Figure 9. MEMRI Auditory ROIS

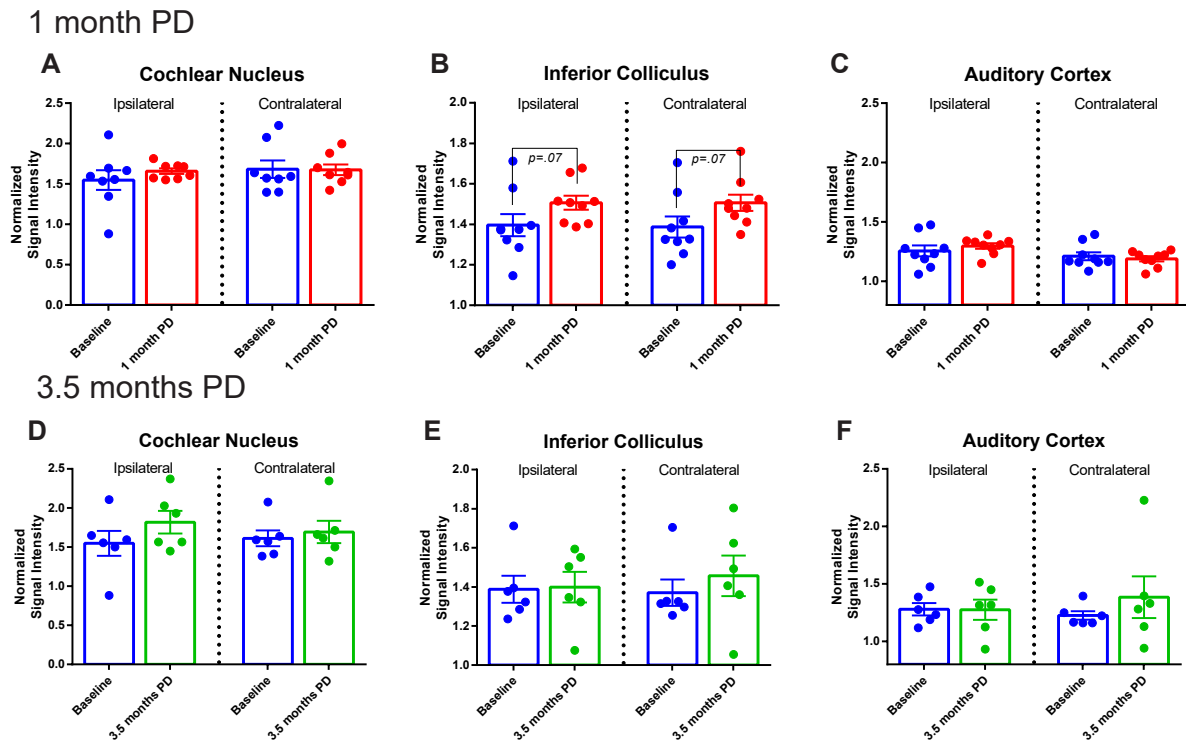


Figure 9. MEMRI measurements in auditory regions of interest. Bilateral spontaneous activity was measured in each ROI at 1 month (A,B,C) and 3.5 months (D,E,F) post damage and compared to measurements taken at baseline. Baseline data at 3.5 months includes only those animals that survived to 3.5 months. Mean normalized signal intensity is plotted as a function of time point for each ROI (+/- standard error of the mean), with data from individual animals shown superimposed. P values indicate results of the Wilcoxon signed rank test. Specific values obtained from this analysis can be seen in Table 2.

Figure 10. MEMRI Representative Images: Non-Auditory ROIs

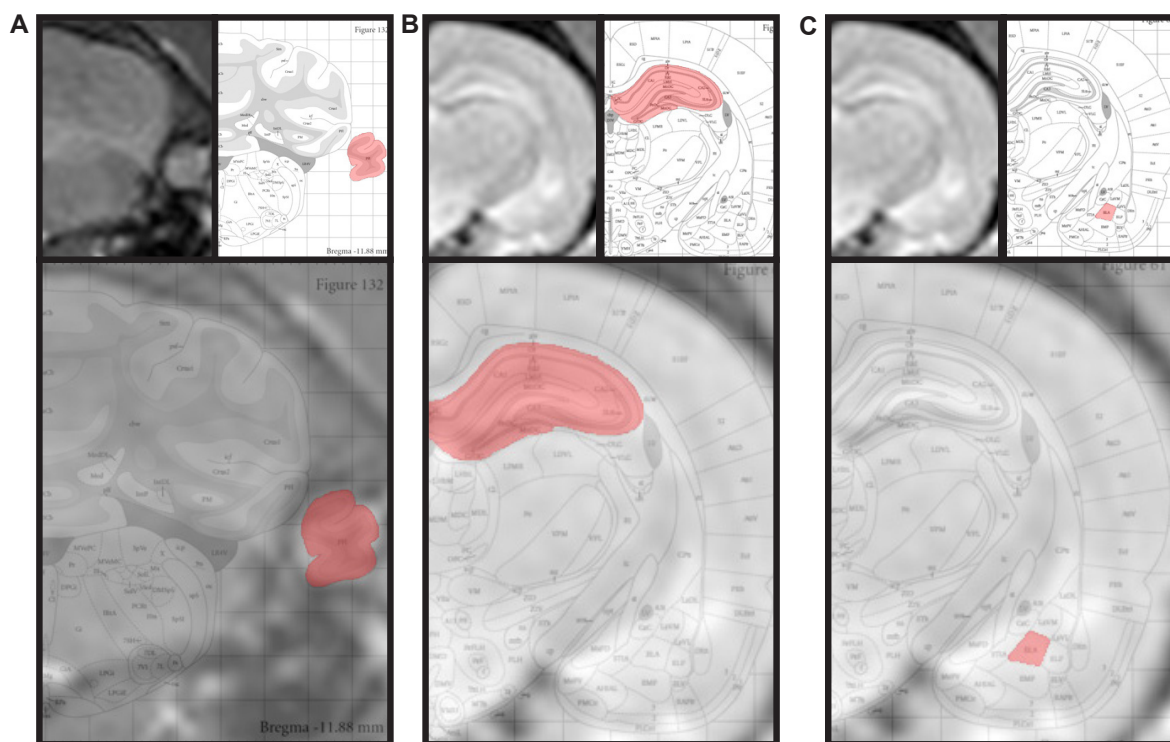
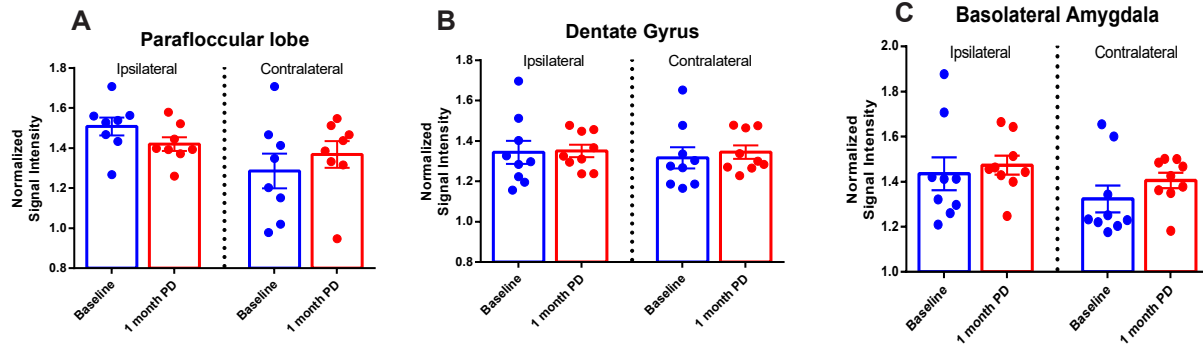


Figure 10. Representative MEMRI images of non-auditory regions of interest. The cerebellar parafloccular lobe (A), hippocampus including dentate gyrus (B) and basolateral amygdala (C) were outlined to measure spontaneous brain activity. Other conventions as in Figure 8.

Figure 11. MEMRI Non-Auditory ROIS

1 month PD



3.5 months PD

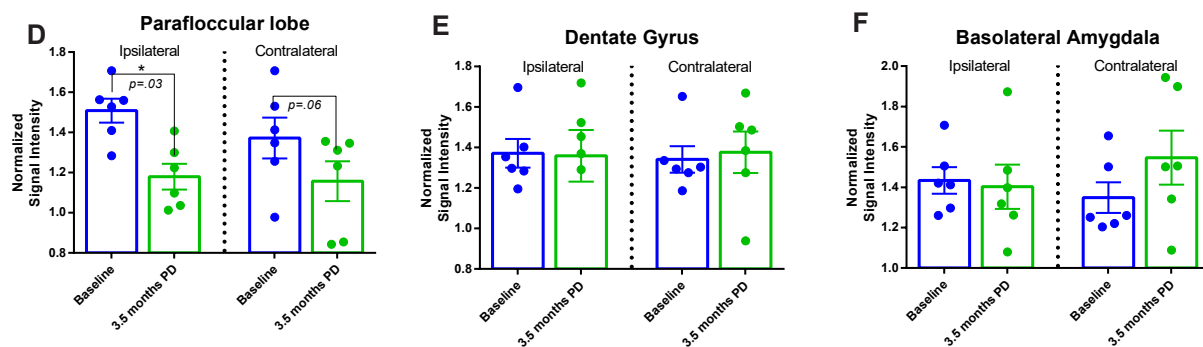


Figure 11. MEMRI measurements in non-auditory regions of interest. Bilateral spontaneous activity was measured in each ROI at 1 month (A,B,C) and 3.5 months (D,E,F) post damage and compared to measurements taken at baseline. Mean normalized signal intensity is plotted as a function of time point for each ROI. Data from each individual animal is also shown in scatter plot style of each graph. There was a bilateral decrease in spontaneous activity of the parafloccular lobe at 3.5 months post damage (IPSI: $p=0.03$, CONTRA: $p=0.06$). Error bars represent standard error of the mean. P values indicated on the graphs indicate results of the Wilcoxon signed rank test. Specific values from this analysis can be seen in Table 2.

Table 2: MEMRI Data Arranged by Time Point and ROI

1 month post damage					3.5 months post damage				
Region of Interest	Ipsilateral to the damaged ear		Contralateral to the damaged ear		Region of Interest	Ipsilateral to the damaged ear		Contralateral to the damaged ear	
	Median of differences	p value	Median of differences	p value		Median of differences	p value	Median of differences	p value
Cochlear Nucleus	0.0456	0.31	0.0087	0.95	Cochlear Nucleus	0.1747	0.44	-0.0466	0.99
Inferior Colliculus	0.1167	0.07	0.0953	0.07	Inferior Colliculus	-0.0153	0.99	0.0634	0.56
Auditory Cortex	0.0316	0.43	-0.0481	0.73	Auditory Cortex	-0.0892	0.84	0.0269	0.69
Parafloccular Lobe	-0.0885	0.15	0.1014	0.20	Parafloccular Lobe	-0.4186	0.03	-0.1591	0.06
Basolateral Amygdala	0.1006	0.73	0.1298	0.16	Basolateral Amygdala	-0.1003	0.56	0.0607	0.56
Hippocampus	0.0399	0.91	0.0326	0.57	Hippocampus	0.0286	0.99	0.0744	0.84

Table 2. MEMRI results arranged by time point and region of interest. Values are reported as median of differences between 1 month post damage and 3.5 months post damage relative to controls. P values are reported as evaluated using the Wilcoxon-Signed Rank Test.

1 Month Post Damage 3.5 Months Post Damage

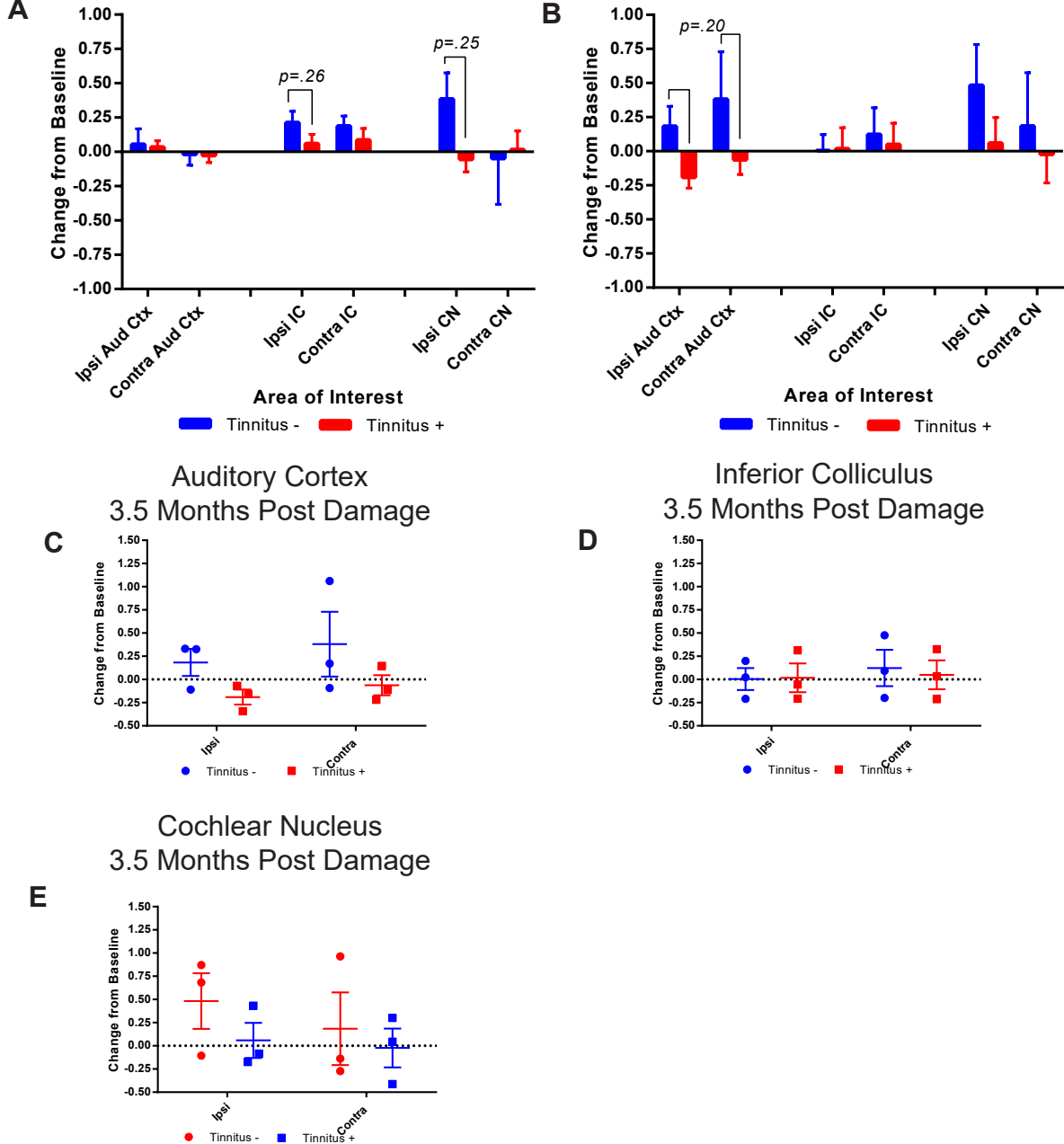


Figure 12. Comparison of changes in spontaneous activity of auditory brain regions in tinnitus positive and tinnitus negative animals as measured with MEMRI. Mean change from baseline at 1 month (A) and 3.5 months (B) post damage is plotted as a function of region of interest. The solid line drawn at 0 is indicative of no change so that positive or negative changes from baseline can be easily seen. Scatter plots for each ROI (C, D, E) at 3.5 months are included due to the high variability at this time point. Error bars indicate SEM.

Figure 13. MEMRI Change from Baseline: Tinnitus + vs Tinnitus - in Non-Auditory Areas of Interest

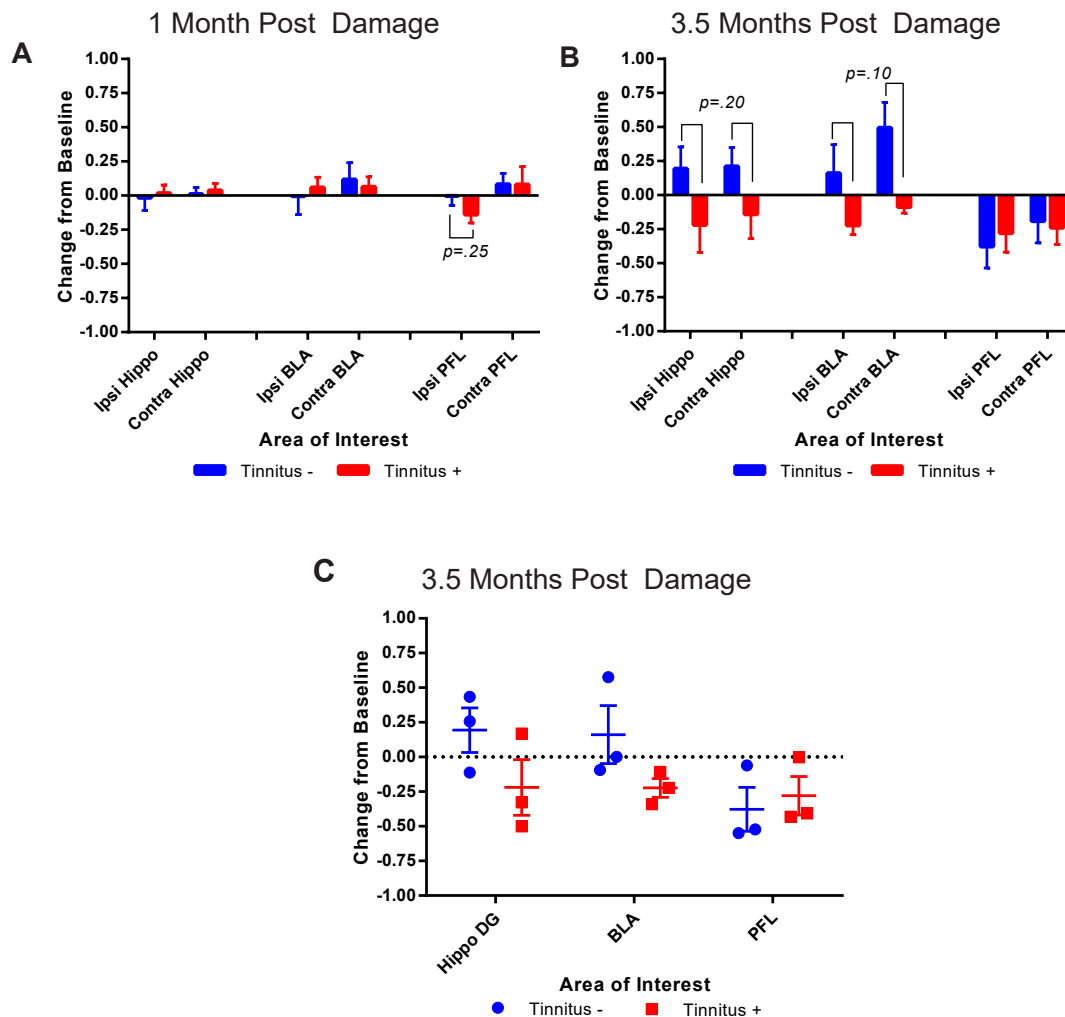
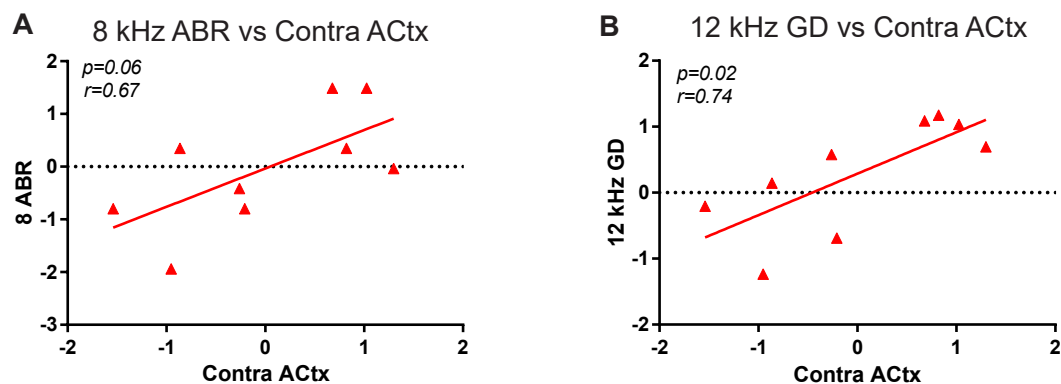


Figure 13. Comparison of changes in spontaneous activity of non-auditory brain regions in tinnitus positive and tinnitus negative animals as measured with MEMRI. Mean change from baseline at 1 month (A) and 3.5 months (B) post damage is plotted as a function of region of interest. The solid line drawn at 0 is indicative of no change so that positive or negative changes from baseline can be easily seen. All other conventions as in Figure 12.

Figure 14. 1 Month Post Damage: Relationships between Auditory Brain Activity, Hearing Loss and Tinnitus Behavior

Direct Pathway



Indirect Pathway

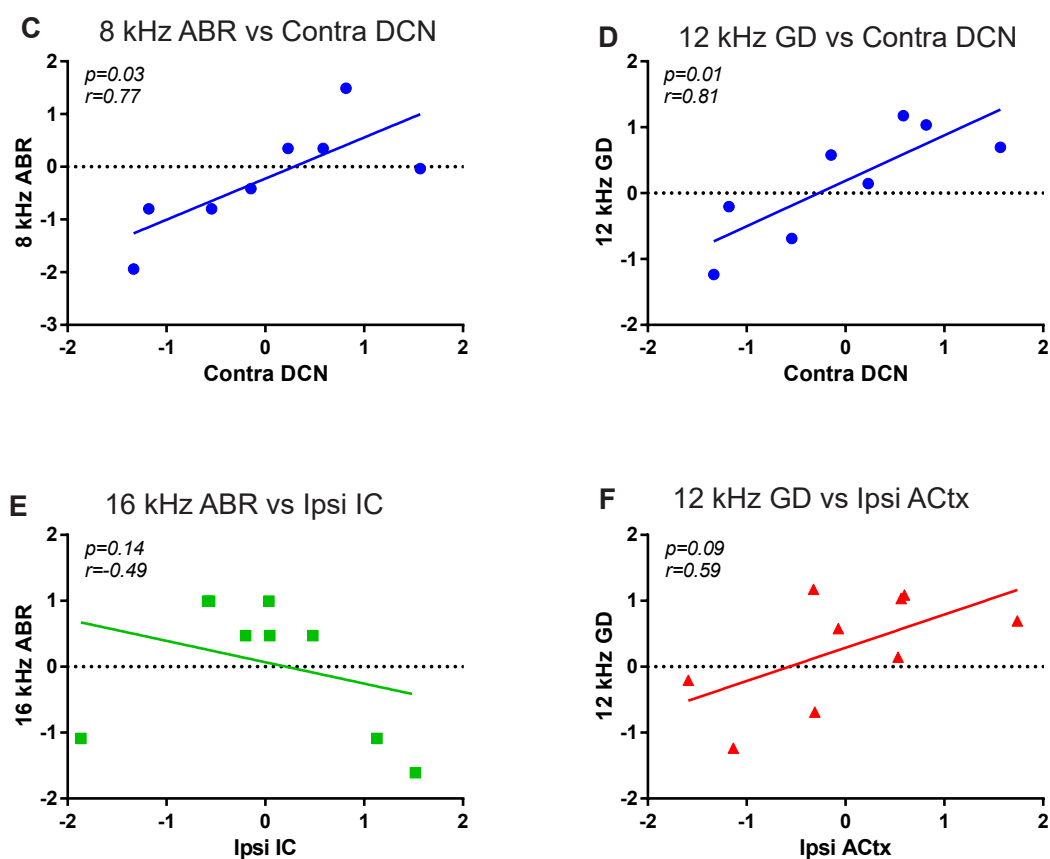
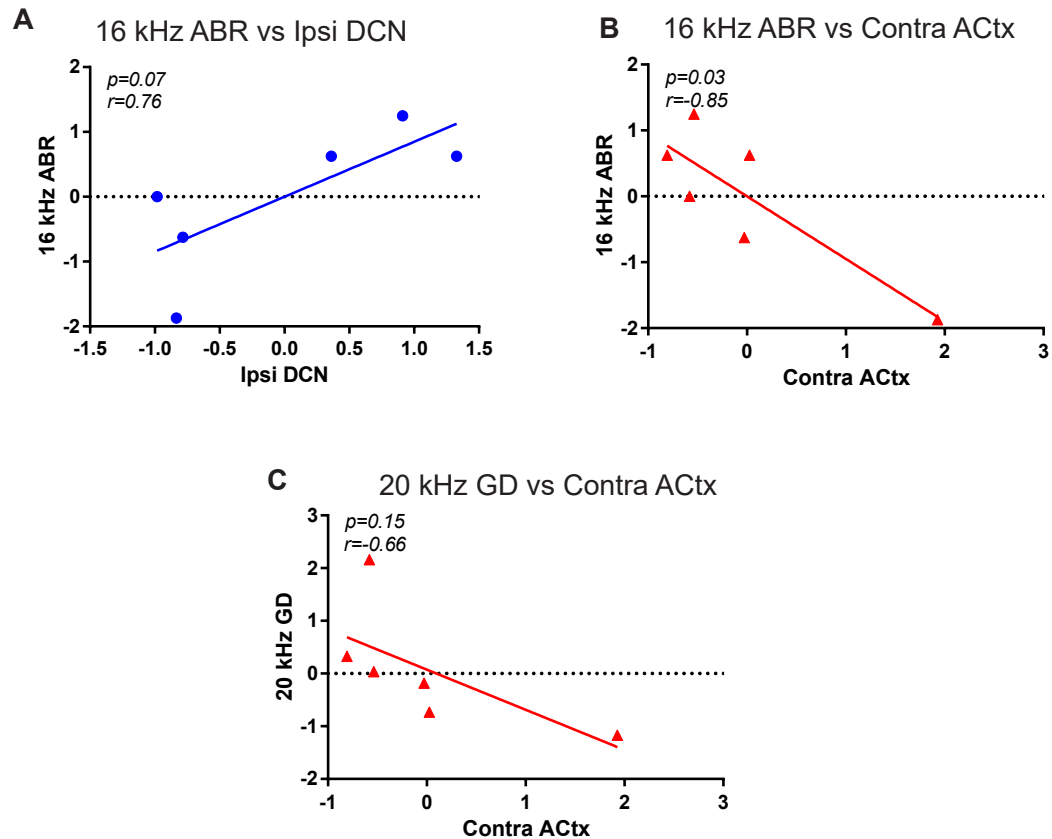


Figure 14. Relationships between auditory brain activity, hearing loss and tinnitus behavior 1 month following damage. Z scores were calculated for each measure and data points represent Pearson correlations. A and B show relationships found in the direct pathway (from the damaged ear) and C-F show relationships found in the indirect pathway.

Figure 15. 3.5 Months Post Damage: Relationships between Auditory Brain Activity, Hearing Loss and Tinnitus Behavior

Direct Pathway



Indirect Pathway

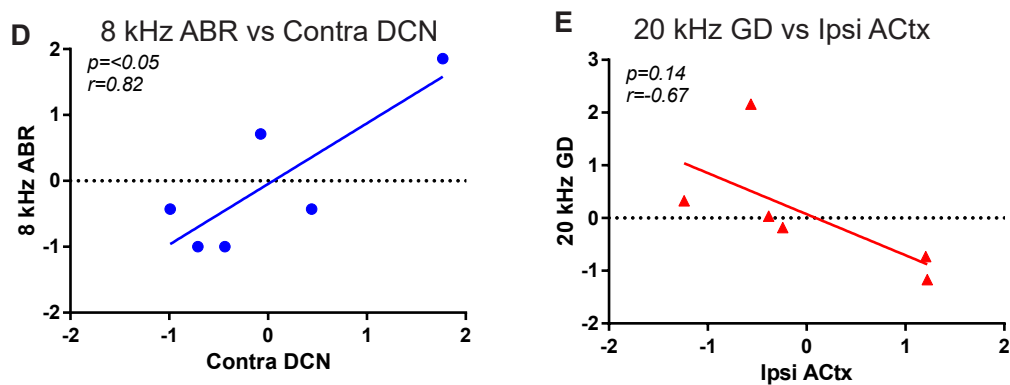
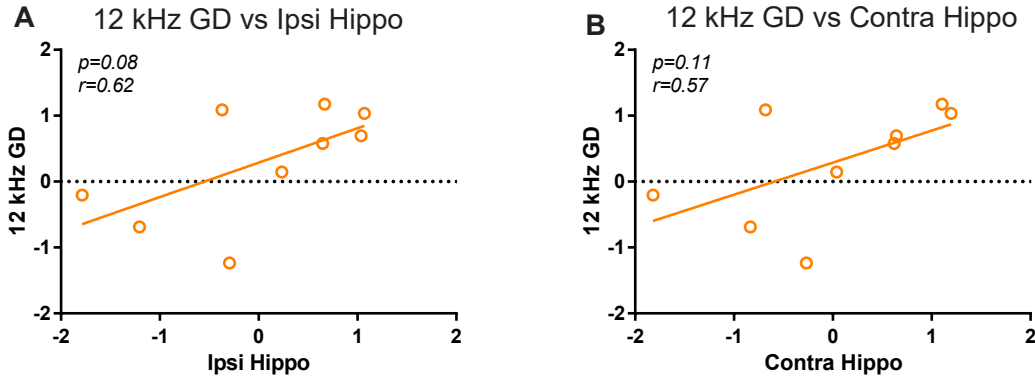


Figure 15. Relationships between auditory brain activity, hearing loss and tinnitus behavior 3.5 months following damage. All conventions as in Figure 14.

Figure 16. 1 Month and 3.5 Months Post Damage: Relationships between Hippocampus Brain Activity and Tinnitus Behavior

1 Month Post Damage



3.5 Months Post Damage

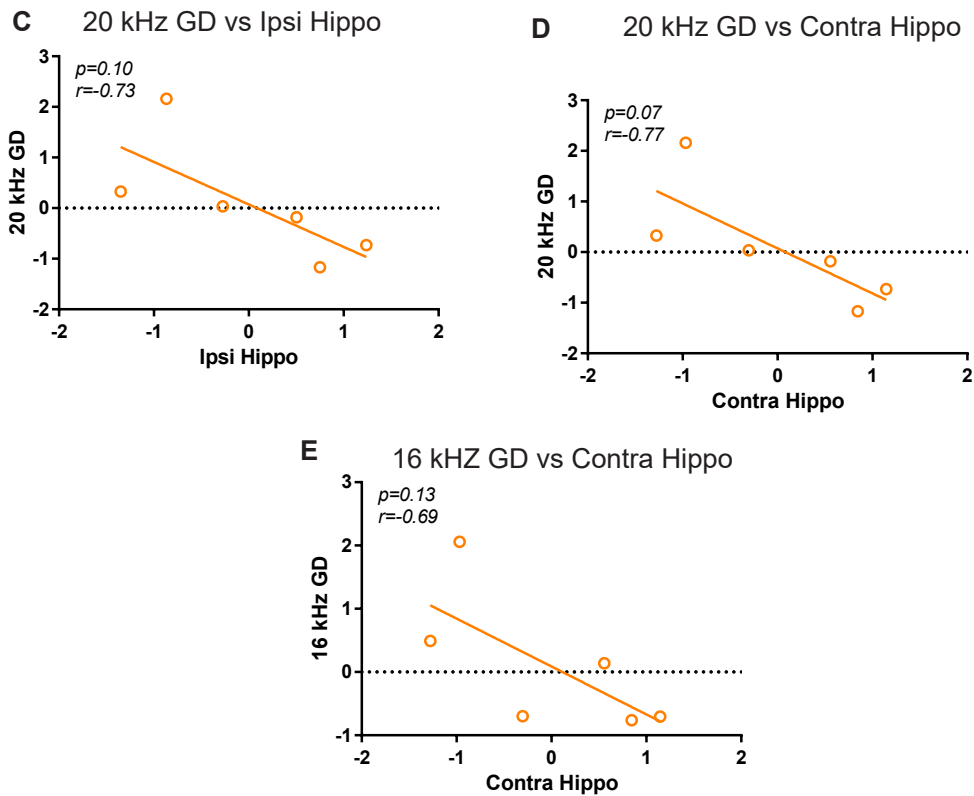
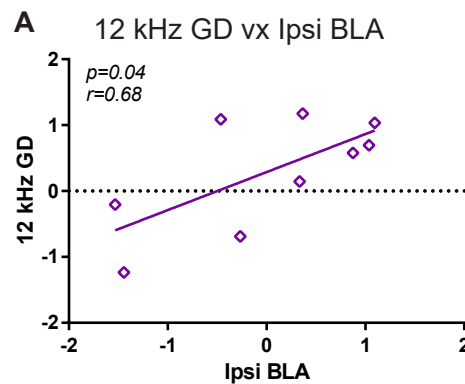


Figure 16. Relationships between spontaneous activity in the hippocampus and tinnitus behavior at 1 month (A and B) and 3.5 months (C-E) post damage. All conventions as in Figure 14.

Figure 17. 1 Month and 3.5 Months Post Damage: Relationships between Parafloccular Lobe and Basolateral Amygdala Brain Activity, Hearing Loss and Tinnitus Behavior

1 Month Post Damage



3.5 Months Post Damage

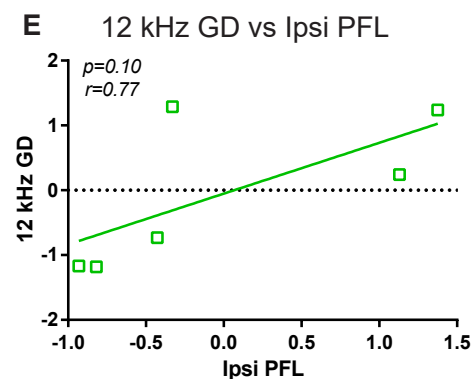
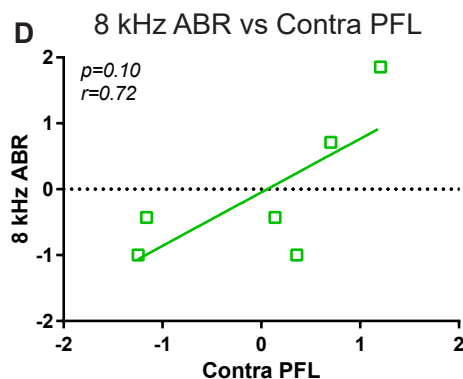
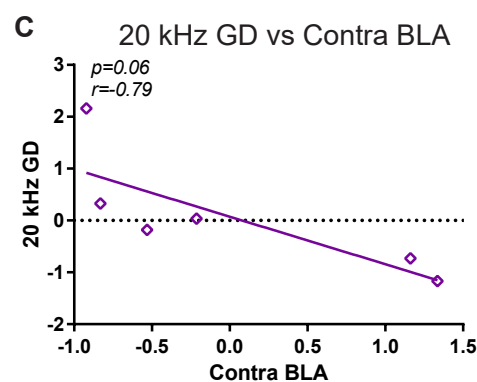
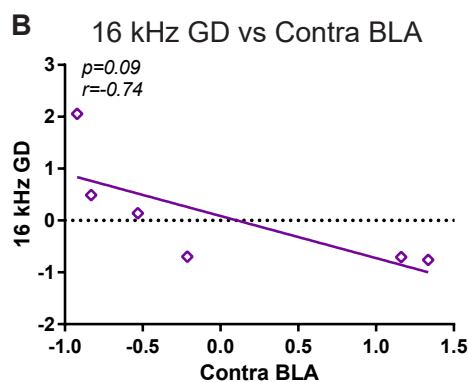


Figure 17. Relationships between cerebellar parafloccular lobe and basolateral amygdala spontaneous brain activity, hearing loss and tinnitus behavior at 1 month (A) and 3.5 months post damage (B-E). All conventions as in Figure 14.

Figure 18. 1 Month and 3.5 Months Post Damage: Relationships between Non-Auditory Brain Activity and Anxiety Behavior

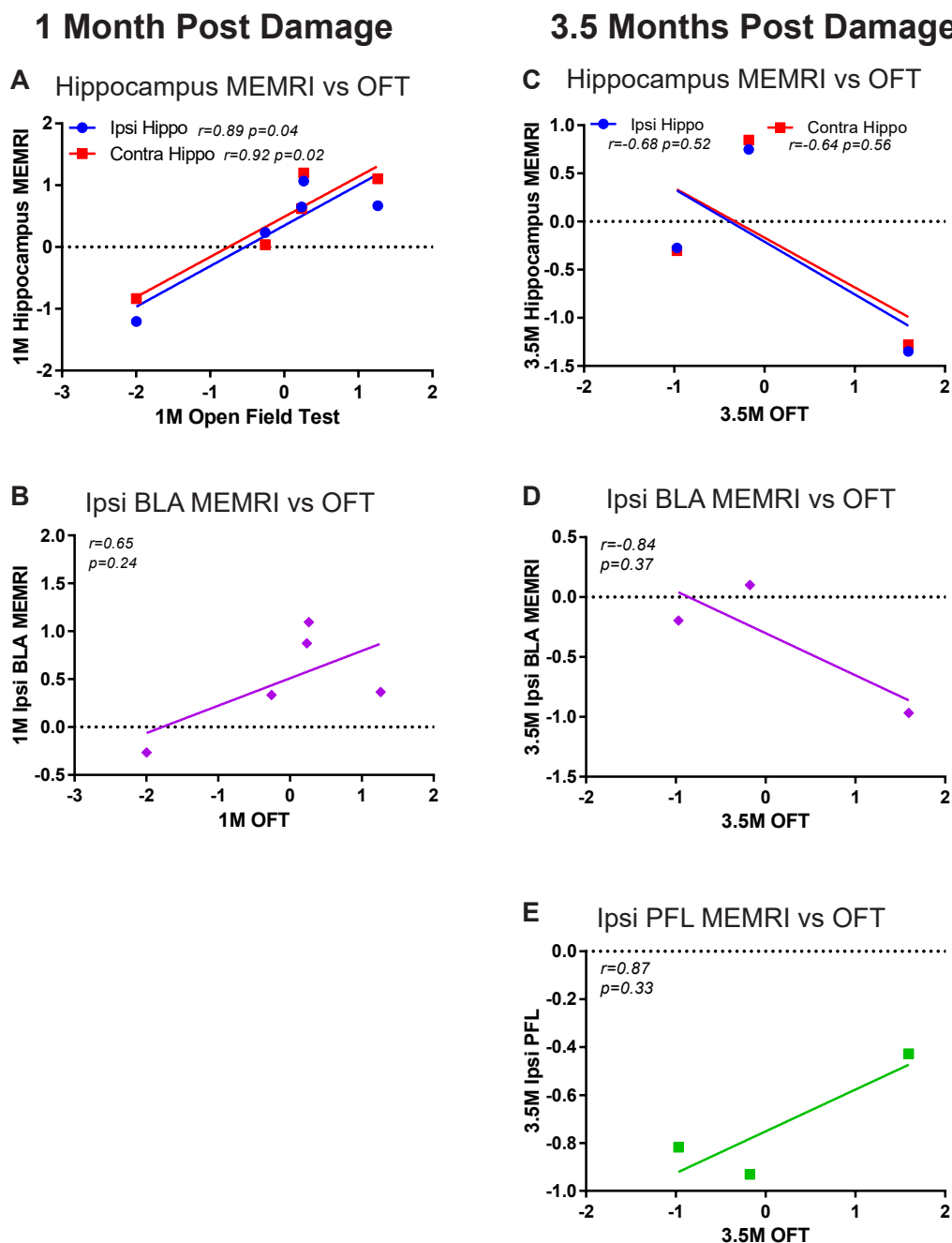


Figure 18. Relationships between non-auditory brain activity and anxiety behavior as measured by open field test. All conventions as in Figure 14.

Figure 19. 1 Month and 3.5 Months Post Damage: Relationships between Auditory Brain Activity and Anxiety Behavior

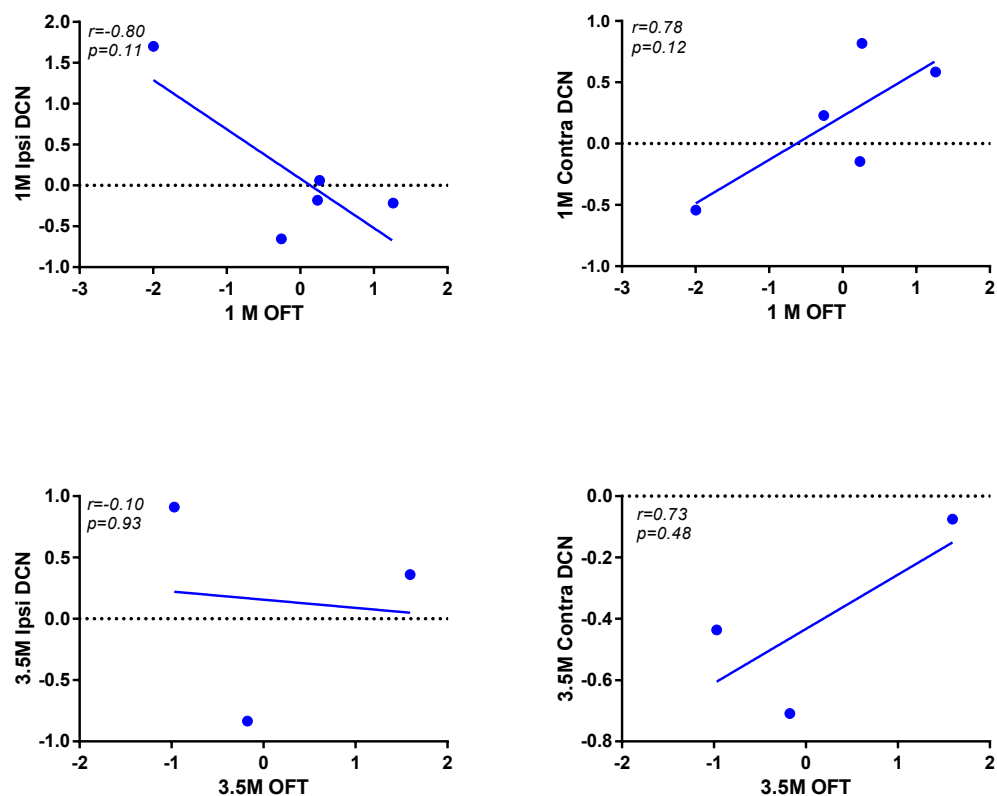


Figure 19. Relationships between auditory brain activity and anxiety behavior as measured by open field test. All conventions as in Figure 14.

Table 3. Relationships Between Spontaneous Brain Activity and Hearing Loss



Positive Correlation
 Negative Correlation

Auditory Brainstem Response

MEMRI region	1 month		3.5 months	
	Side, Frequency	r, p value	Side, Frequency	r, p value
Cochlear Nucleus	Contra, 8 kHz	r=0.77, p=0.03	Contra, 8 kHz Ipsi, 16 kHz	r=0.82, p=0.05 r=0.76, p=0.07
Inferior Colliculus	Ipsi, 16 kHz	r=-0.49, p=0.14		
Auditory Cortex	Contra, 8 kHz	r=0.67, p=0.06	Contra, 16 kHz	r=-0.85, p=0.03
Parafloccular Lobe			Contra, 8 kHz	r=0.72, p=0.10

Table 3. Relationships between spontaneous brain activity (MEMRI) and hearing loss (ABR) 1 month and 3.5 months post damage. Ipsi = Ipsilateral to the damaged ear, Contra = Contralateral to the damaged ear. Green = Positive correlation, Red = Negative correlation. Pearson Correlation, significance $p < 0.05$.

Table 4. Relationships Between Spontaneous Brain Activity and Tinnitus Behavior

 Positive Correlation  Negative Correlation

Gap Detection

MEMRI region	1 month		3.5 months	
	Side, Frequency	r, p value	Side, Frequency	r, p value
Cochlear Nucleus	Contra, 12 kHz	r=0.81, p=0.01		
Auditory Cortex	Bilateral, 12 kHz	Ipsi (r=0.59, p=0.09) Contra (r=0.74, p=0.02)	Bilateral, 20 kHz	Ipsi (r=0.67, p=0.14) Contra (r=-0.66, p=0.15)
Parafloccular Lobe			Ipsi, 12 kHz	r=0.77, p=0.10
Basolateral Amygdala	Ipsi, 12 kHz	r=0.68, p=0.04	Contra, 16 kHz Contra, 20 kHz	r=-0.74, p=0.09 r=-0.79, p=0.06
Hippocampus	Bilateral, 12 kHz	Ipsi (r=0.62, p=0.08) Contra (r=0.57, p=0.11)	Contra, 16 kHz Bilateral, 20 kHz	r=-0.69, p=0.13 Ipsi (r=-0.73, p=0.10) Contra (r=-0.77, p=0.07)

Table 4. Relationships between spontaneous brain activity (MEMRI) and tinnitus behavior (gap detection) 1 month and 3.5 months post damage. Ipsi = Ipsilateral to the damaged ear, Contra = Contralateral to the damaged ear. Green = Positive correlation, Red = Negative correlation. Pearson Correlation, significance $p < 0.05$.

Chapter 4: Conclusions and Summary

4.1 Purpose

The experiments reported here were designed to elucidate the mechanisms underlying brain changes as a result of peripheral sound damage that may or may not be related to tinnitus. Doublecortin (DCX) was used to identify brain plasticity that occurs in the dorsal cochlear nucleus, cerebellar parafloccular lobe and hippocampal dentate gyrus following sound damage. Labeling was compared in control and sound damaged animals approximately two weeks after damage. Manganese-enhanced MRI (MEMRI) was used to measure changes in spontaneous brain activity (SA) that occurs as a result of exposure to damaging sound, hearing loss and tinnitus. Measurements were taken at 1 month post damage and 3.5 months post damage in the same animal allowing us to observe change over time. Changes in SA were compared over time in 3 auditory (dorsal cochlear nucleus, inferior colliculus, auditory cortex) and 3 non-auditory (cerebellar parafloccular lobe, hippocampal dentate gyrus, basolateral amygdala) brain regions. For both experiments, gap detection testing was used to identify tinnitus behavior and comparison to control data enabled us to separate animals into tinnitus subgroups. Because we are interested in both auditory and non-auditory outcomes of tinnitus, we measured anxiety behavior in a subgroup of animals during the MEMRI study. The combination of DCX labeling and MEMRI following the same sound damage paradigm, enables the comparison of two techniques addressing both a cell specific and activity specific neural correlate of tinnitus. This study addresses changes over time and spans 6 brain regions, including both auditory and non-auditory areas. Importantly, the outcome of each measure can be used to identify relationships that exist between hearing loss, tinnitus behavior, and brain plasticity.

4.2 Comparison of DCX and MEMRI One Month After Damage

Similar study designs were used in our DCX and MEMRI experiments (1 month post damage) to allow comparisons between cell-specific and region specific measurements of CNS neuroplasticity that occur following sound damage.

Tinnitus Behavior

The assessment of gap detection behavior was successful in identifying tinnitus subgroups within our sound damaged animal populations. Our method of tinnitus group assignment considered baseline and post damage performance as well as the performance of control animals. Animals in the DCX cohort were split into three groups: tinnitus positive (n=4), tinnitus negative (n=5), and improved (n=3). The last group consisted of an unexpected group of animals that displayed significantly improved gap detection performance. Animals in the MEMRI cohort were split into two groups: tinnitus positive (n=6) and tinnitus negative (n=4). While we did have animals in this group with improved performance, there were not enough tinnitus negative animals to split the group into an additional subgroup. Comparing gap detection results from two different cohorts of animals exposed to the same damaging sound stimulus yielded different ratios of animals identified as tinnitus positive. This finding is not surprising as many sound induced tinnitus models have reported variable behavioral outcomes often identifying tinnitus in about half of damaged animals. The variability seen in tinnitus animal models closely mimics the variability seen in clinical population. As explained in previous chapters and expanded on below, carefully grouping damaged animals into tinnitus subgroups can have a dramatic effect on interpretation of CNS correlates of tinnitus.

Hearing Loss

While tinnitus behavior may or may not be evident following sound damage, we do not see the same variability in observed hearing loss post damage. Our mild sound damage paradigm produced significant threshold shifts in damaged animals of both the DCX (shifts at 8, 11.3, 16, 22.6 & 32 kHz) and MEMRI (shifts at 4, 8, 11.3, 16 & 32 kHz) cohorts. None of the sound damaged animals from either group had threshold increases greater than 50dB. Two conclusions can be made from this comparison: hearing loss produced by this sound damage paradigm is consistent and repeatable, and this sound damage is mild enough that it does not induce hearing loss so severe that it interferes with an animal's ability to detect stimuli presented (at 60 dB) during gap detection testing.

Dorsal Cochlear Nucleus

When all sound damaged animals were grouped together, we observed no differences in DCX labeling (relative to controls) or MEMRI signal (relative to baseline). However, when animals were sorted according to tinnitus group, tinnitus negative animals had increased high frequency DCX label (relative to tinnitus positive and improved groups) and increased MEMRI signal (relative to tinnitus positive) in the *ipsilateral* DCN. Interestingly, high frequency DCX labeling was decreased in the *contralateral* DCN of tinnitus negative animals (relative to tinnitus positive). In a recent study, Brozoski and colleagues (2017) suggest that DCX upregulation could be a sign of cells seeking to maintain or reinitiate homeostatic equilibrium. While they observed no change in DCX label in the DCN of animals with no behavioral evidence of tinnitus, they posit that upregulation may occur via a MAP2-K2p mechanism (membrane potential stabilization) as DCX overexpression in vitro has been shown to increase MAP2 (Brozoski et al 2017). If this is correct, DCX should be enhanced in tinnitus negative animals relative to tinnitus positive animals, that is what we observed in the ipsilateral high

frequency DCN. This suggests that upregulation of DCX expression is occurring in UBCs which may serve a protective role in the brains of individuals exposed to damaging sounds that do not go on to develop tinnitus. We also observed an increase in MEMRI signal (spontaneous activity) of the ipsilateral DCN in tinnitus negative animals. Increased brain activity (observed with MEMRI) could be a result of increased excitatory UBC activity (observed with DCX labeling) in the ipsilateral DCN.

Cerebellar Parafloccular Lobe

Sound damaged animals showed bilateral increases in DCX label (relative to controls) when all animals were grouped together while no changes in MEMRI signal (relative to baseline) were observed. When animals were sorted into tinnitus groups it became obvious that it was the tinnitus negative animals that were pulling up the average label of DCX in the group of sound damaged animals as there were no statistical differences observed in tinnitus positive animals (relative to controls). While not significant, when MEMRI animals were sorted according to tinnitus status we observed a trend toward decreased activity in the ipsilateral PFL of tinnitus positive animals (relative to tinnitus negative). Increased DCX label in the PFL following sound damage has been reported previously (Bauer et al 2013b) while MEMRI studies have shown increased activity of the PFL (Brozoski et al 2007). Observations with DCX labeling post damage as reported here support other studies in that DCX labeling was increased after damage; however these findings were reflective of animals that did not develop tinnitus. Importantly, we did not observe changes in MEMRI signal post damage. This suggests that time plays a role in changes reported in the PFL and that DCX may be labeling synaptic processes that differ from altered spontaneous activity (measure dependent on action potentials).

Hippocampal Dentate Gyrus

DCX labeling was significantly decreased in bilateral dentate gyri of sound damaged rats (relative to controls) while no changes in MEMRI signal (relative to baseline) were evident when all sound damaged animals were grouped together. Grouping animals based on tinnitus groups revealed significant bilateral decreases in DCX label of all tinnitus groups (relative to controls) while no changes were evident when tinnitus groups were compared to each other. While not statistically significant, there was a trend toward decreased hippocampal activity, as measured by MEMRI, in tinnitus positive animals (relative to tinnitus negative). Taken together these results suggest that decreased DCX labeling in the hippocampus results from hearing loss regardless of tinnitus status. They also suggest that DCX may have a different role in the hippocampal granule cells (neurogenesis) than it does in the UBCs of the DCN and cerebellum (broad plasticity or changes in glutamatergic activity).

4.3 Relationships Between Measured Variables

To our knowledge there have not been any published studies investigating potential relationships between the many measured variables commonly examined in tinnitus studies. While it is known that tinnitus is associated with hearing loss and other forms of peripheral auditory damage, the strength and direction of these relationships is not clear. Our study design, in which we measure many different outcomes in the same animals over time, enabled the identification of correlations that occur following exposure to damaging sound. A correlation between two events does not mean that a causal relationship exists. Rather, strong relationships identified by correlation analysis provide a framework for which tinnitus

investigators can utilize in determining future experiments. With a few exceptions, strong positive relationships, identified between spontaneous brain activity and hearing loss, suggest that perhaps the tinnitus percept is not necessary to modify CNS activity following damage to the periphery. Interestingly, strong positive relationships observed between spontaneous brain activity and tinnitus behavior evident at the early time point (1 month post damage) became negative at the later time point (3.5 months post damage) in 1 auditory (auditory cortex) and 2 non-auditory (basolateral amygdala and hippocampus) brain regions. It has been proposed that limbic brain regions (including both amygdala and hippocampus) may serve to block the auditory cortex from perceiving the tinnitus percept in cases where tinnitus does not occur (Rauschecker et al 2010). Perhaps our correlation results are indicative of this occurrence. While these three brain regions play differing roles in brain function, this trend warrants further investigation which may result in a greater understanding of altered tinnitus percept in association with altered brain activity over time.

4.4 Limitations

While our experiments generated a significant amount of data that aids in a better understanding of tinnitus, our work is not without limitations. Sample sizes were limited, especially once we sorted animals according to tinnitus behavior. As a result, we utilized nonparametric statistics which have the advantage of not being dependent on parametric assumptions but lack large statistical power. This does not suggest that our results lack importance but rather that a larger sample size would allow for a better representative sampling of the population thereby increasing the strength of our findings.

While numerous studies employ the use of “protein markers” immunohistochemical labeling can sometimes be misleading as we cannot be certain the presence of a specific protein infers a certain function (e.g. plasticity). Therefore, results should be interpreted carefully.

Measuring tinnitus behavior in animals has proved to be quite challenging in the field of auditory research and is not without limitations, as discussed more fully in previous chapters. While we optimized the gap startle test by modifying our analysis methods, we cannot be certain that impaired ability to detect a gap of silence indicates tinnitus. We have observed that results are dependent on how animals are grouped (e.g. all sound damaged animals together, various tinnitus subgroups). Therefore utilizing some method to identify tinnitus behavior in a group of animals that underwent the same sound damage is necessary for differentiating between changes resulting from hearing loss *alone* and changes resulting from *both* hearing loss and tinnitus.

MEMRI use in auditory research is becoming more common due to the ability to bypass confounds of noise produced during the scan. In our experiments, animals were injected with MnCl_2 , returned to their home cages that were housed inside a sound attenuated booth for 24 hours. Although we took all precautions to ensure a quiet environment during manganese uptake, it is impossible to ensure that there was not noise generated by the animal over the 24 hour uptake period. It should be noted that signal intensity as measured by MEMRI post damage was compared to baseline signal intensity, thereby cancelling out any changes resulting from self-produced noise.

4.5 Future Work

The work presented here has provided a significant contribution to existing tinnitus research, but there is still much to be done. Tinnitus is a complex, multifaceted disorder and there continue to be big gaps in the existing knowledge of how, where and when tinnitus is generated and subsequently where to focus in order to develop effective treatments.

Measurements of DCX labeling in animals at 3.5 months post damage would add an interesting piece to the puzzle of tinnitus. There are no published reports on the pattern of DCX labeling over time. Because we know that DCX is expressed in glutamatergic unipolar brush cells (UBCs) in the DCN and PFL and granule cells of the hippocampus, knowing how expression changes over time post damage in these specific cell types would be useful. This would give us insight into cell-specific changes that occur at both acute and chronic time points following damage.

Co-labeling with DCX and BrdU in the hippocampus would be another interesting follow up study. Studies that have been done in the PFL and DCN to disentangle the relationship between DCX label and neurogenesis report that, at least in these two non-neurogenic regions, DCX does not appear to be labeling newly generated cells. Since the hippocampus is known location of adult neurogenesis, determining the role of DCX here would be informative. The presence of cells that co-label with DCX and BrdU in the hippocampus that change as a result of sound damage would indicate that altered DCX post damage means altered neurogenesis.

The use of the gap detection paradigm in control and sound damaged animals allowed us to group our animals into tinnitus subgroups based on their gap detection performance. In

our DCX cohort of animals, we identified a small group of animals (improved, $n = 3$) that displayed significantly improved performance post damage relative to their baseline performance and controls when the background stimulus was centered at 12 kHz. While this group does not appear to have developed tinnitus, sorting these animals into a unique group yielded interesting relationships between gap detection performance at 12 kHz and brain activity in both auditory and non-auditory brain regions at 1 month post damage. Therefore, it would be useful to generate more sound damaged animals for two reasons; to determine if we get more animals displaying similar improvement in performance, to look more closely at the identified relationships to determine causality.

Our study design allowed for extensive correlation analysis whereby we identified many strong relationships between hearing loss, tinnitus and anxiety. Correlation does not imply causation, but significant relationships existing between two variables definitely warrant further study.

Tinnitus often occurs concomitantly with depression or anxiety. While this study does investigate changes in limbic brain regions, we did not observe any differences in anxiety behavior between any groups when compared to baseline behavior. This does not mean that those behaviors aren't present or that they wouldn't be present following peripheral damage in the clinical population. Instead it suggests that our measure of anxiety behavior may lack sensitivity within this specific model. Additionally, individuals in the human population encounter many more potential stressors throughout life than a typical laboratory animal. Therefore, measuring affective behaviors is inherently difficult in animals as depression and anxiety in an animal likely manifest differently than in a human. Exposing an animal to known

stressors prior to sound damage may be necessary to induce measureable affective behavior in an animal model of tinnitus.

4.6 Contribution and Relevance

Tinnitus affects approximately 50 million individuals in the United States (ATA.org) with a subgroup reporting a severe decrease in quality of life that could potentially lead to suicide (Roberts et al 2010). Despite the fact that this is a widespread problem, the complexity of the disorder continues to hinder a complete understanding of the mechanisms underlying tinnitus (Seidman et al 2010). The absence of a complete picture of how tinnitus is manifested and maintained in the CNS continues to hinder the development of effective treatments. The findings reported here reveal information about the timeline of peripheral injury (sound damage) to tinnitus onset and changes that take place in six different brain regions encompassing both auditory and non-auditory brain regions. The strength of our experimental design allowed us to identify numerous relationships that exist between variables measured by many tinnitus investigators. These relationships provide a foundation from which future experiments can be developed to further enhance our understanding of chronic tinnitus.

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